

# Effects of aromatase inhibitors on aromatase in breast

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**Doctor of Medicine  
The University of Edinburgh  
2009**

*To my parents for their support and love,*  
*To Venkat for his patience and understanding,*  
*To my sister for her support and enthusiasm*

## **Acknowledgements**

I would like to thank Prof WR Miller for the inspiration, guidance and for acting as my supervisor.

Mr JM Dixon for his enthusiasm and clinical supervision.

Prof Ian Mason for his assistance and advice.

Martin Hulme and Forbes Howie who patiently taught me the techniques.

Alexi Larinov for conducting the PCR experiments.

Statistics department, University of Birmingham for analysing the data and performing the statistics.

I would like to thank all the patients who provided tissue samples for the study.

Finally I would like to acknowledge Novartis for providing the research fellowship that enabled me to conduct the project.

## Declaration

I hereby declare that I am the sole author of this thesis and that all work within is my own, except where work carried out in collaboration with other individuals, who are appropriately credited. I authorise the library of the University of Edinburgh to lend this thesis to other institutions or individuals for the purpose of research.

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Dated: 24/4/09



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## **AIMS**

- To determine the effects of different aromatase inhibitors on aromatase activity
- To assess aromatase activity in response to primary endocrine therapy

These aims are addressed sequentially by:

[i] Assessing validation and reproducibility of the methodology to determine aromatase activity.

[ii] Determining the potency of different aromatase inhibitors – type I (exemestane, formestane) and type II (aminoglutethimide, letrozole, anastrozole, fadrazole) in a mammary adipose tissue fibroblast culture model and the time scale needed for the effects.

[iii] Measuring aromatase activity before and during primary endocrine therapy in malignant and nonmalignant tissue specimens from postmenopausal women with breast cancer.

[iv] Assessing changes in molecular expression associated with letrozole treatment by measuring mRNA, the type of transcripts expressed and the changes in expression of transcripts before and after treatment.

## ABSTRACT

Certain breast cancers require oestrogen for their continued growth. In postmenopausal women, in whom most breast cancers arise, oestrogen is primarily synthesised in peripheral sites such as adipose tissue, muscle and within the breast cancers themselves. As these sites are not amenable to surgical removal the concept of inhibiting oestrogen biosynthesis with drugs is an attractive treatment option. Consequently, aromatase inhibition has become a major treatment strategy for postmenopausal women with oestrogen-dependent breast cancer.

The aims of this study were to determine the effects of both type I (exemestane, formestane) and type II aromatase inhibitors (letrozole, anastrozole, fadrazole, aminoglutethimide) on aromatase activity. Studies based on invitro incubations of cultures of mammary adipose tissue fibroblasts demonstrate that the new aromatase inhibitors are highly effective, with  $IC_{50}$  values in the lower nanomolar range (1 - 50 nm). Aromatase inhibitors such as letrozole (100nm) produced almost total inhibition of aromatase activity (97%) demonstrating its remarkable potency.

The fibroblast cultures were induced to display aromatase activity by preincubation with either dexamethasone or dibutyryl cyclic AMP. The induced aromatase activity showed classic sensitivity to aromatase inhibitors. However, if aromatase inhibitors were included in the culture media during the preincubation period, enhanced activity was displayed with type II inhibitors (letrozole, anastrozole, aminoglutethimide) but not with type I inhibitors (exemestane, formestane). This effect may reflect the possibility of developing resistance with the long term use of type II inhibitors and suggests the sequential use of type II followed by type I aromatase inhibitors in the treatment of breast cancer.

Sixteen postmenopausal patients with large (>3cm), oestrogen receptor positive (>20 fmol/mg cytosol protein), primary breast cancers (stages T2, T3, N0, N1) were treated with aromatase inhibitors - letrozole, anastrozole and exemestane prior to surgery and aromatase activity was measured (invitro) before and after 3 months of treatment. Significant reduction in the aromatase activity was found following treatment in

malignant tissues. The changes following treatment with letrozole, anastrozole and exemestane were statistically significant between pre and post treated tumour samples ( $p=0.017$ ,  $p=0.01$ ,  $p<0.05$ ). This demonstrates the potency of the third generation aromatase inhibitors in reducing aromatase activity in tumour tissues. The nonmalignant tissues had a variable response with some displaying enhanced aromatase activity following treatment.

Molecular changes underlying the mechanism associated with letrozole treatment was also studied. The coding part of the aromatase gene was expressed in all the samples studied. Possible induction of the exon I variant I.1 and I.4 following treatment was demonstrated, although this was not totally reproducible. These findings suggest that there may be a switch in the regulatory mechanism of aromatase activity from normal breast tissue to cancerous tissue.

This study has demonstrated the effects of aromatase inhibitors on aromatase in both response and resistance to therapy. The new generation of aromatase inhibitors are potent agents capable of profoundly influencing endocrine events within the breast. Paradoxical response observed with type II aromatase inhibitors under certain circumstances may have important implications for their long term and sequential use.

## INTRODUCTION

The epidemiology of breast cancer, anatomy and physiology of breast and relevance of steroid hormones are discussed. The biology of oestrogen as applied to the development and progression of breast cancer has been reviewed.

Further consideration has been given to

- [i] The patterns of endogenous oestrogen within the breast.
- [ii] The sources by which the breast and tumours may be supplied with oestrogen and mechanisms by which oestrogen may exert its action.
- [iii] The methods of hormonal deprivation, the molecular events which may follow oestrogen deprivation leading to hormone independence.
- [iv] The role of aromatase and aromatase inhibitors.

## BREAST CANCER

Breast cancer is the most common female cancer, with a worldwide yearly estimate of one million new cases and accounts for 22% of all cancers in women [Shibuya K]. Incidence rate varies between populations, with the highest rates in Western countries (over 100 cases per 100,000 women) and the lowest in Asian countries (10 - 15 per 100,000 women). In the UK, there are over 38,000 new cases of breast cancer diagnosed each year and accounts for 17% of all cancer deaths [National Statistics, 2001]. In the United States, approximately 180,000 new cases of breast cancer are diagnosed annually and accounts for 48,000 deaths per year [Jemal A]. Approximately 1% of breast cancers occur in males with about 250 cases diagnosed each year in the UK [National Statistics, 1999].

The incidence of breast cancer increases with age, with 80 % of cases occurring in postmenopausal women. Several well-established risk factors are associated with the development of breast cancer, primarily age and female sex [Fentiman IS].

Breast cancer is the most common cause of cancer related mortality among women [Hortobagyi GN]. The age-standardised mortality has been increasing steadily ever since the 1960s, suddenly in the late 1980s it flattened out and then during the 1990s it decreased substantially [Beral V]. Data has revealed a significant improvement in survival during the last 5-year period (1989-1994) compared with the previous 15 years [National Statistics, 2001]. The decrease strongly suggests that the improvement is chiefly due to the changes in the way breast cancer has been diagnosed and treated. Peto et al have suggested that the reduced mortality was due to the use of tamoxifen and

adjuvant treatment, whereas Tabar et al have attributed it to widespread screening with mammography. Although overall survival has improved with earlier diagnosis, breast cancer still represents a leading cause of cancer-related death.

During the last two decades, significant advances have been made including the development of early detection methods, less radical surgery, new adjuvant therapies and improved supportive care [Botha JL]. Management of breast cancer has traditionally been based on the stage of the disease: early disease stages I and II and advanced stages III and IV. The treatment of patients with early breast cancer is curative and has two main aims - to achieve local disease control and to treat micrometastatic disease. The treatment of patients with advanced disease is to control the disease while maintaining the quality of life. Local disease control is achieved by surgery to breast and axilla and adjuvant therapy with either cytotoxic drugs or endocrine agents or both is recommended for patients with node-positive disease [McArdle CS]. Radical surgery has been replaced by breast conservation surgery as both have similar survival rates [Veronesi U, 1981]. For locally advanced disease multimodality therapy is recommended with the sequence of chemotherapy, surgery and radiotherapy largely dependent on the operability of the primary disease. Wide ranges of systemic, local and supportive therapies are available for the palliation of metastatic breast cancer.

Successful delivery of effective adjuvant systemic therapy acts as a complement to surgical management of breast cancer [Peto R]. The absolute benefit will be a function of the patient's risk of relapse at the time of diagnosis. The Early breast cancer Trialists Collaborative Group (EBCTCG) has provided the foundation for consensus statements regarding selection of cases in which adjuvant therapy is likely to be beneficial. The



National Surgical Adjuvant Breast Project (NSABP) protocols B-13 and B-14 documented the survival advantages associated with adjuvant chemotherapy and endocrine therapy for patients who have node negative, endocrine resistant or endocrine sensitive disease. Chemotherapy is now recommended for high-risk endocrine responsive disease and for any endocrine resistant breast cancer that is deemed appropriate for systemic treatment. Patients who have node positive breast cancer have the largest magnitude of benefit from adjuvant therapy [EBCTCG, 2005]. The toxicity of adjuvant therapy must be carefully, balanced against the relapse risk. Genetic profiling and assignment of a recurrence score via the Oncotype DX test can be helpful in determining the benefit from adjuvant therapy [Olivotto IA].

Although the ultimate goal of treatment remains improved survival, increasing emphasis is now placed on less morbid treatments and enhancing quality of life. The resurgence of interest in endocrine strategies can be attributed to low toxicity profiles of effective hormonal agents. This thesis has mainly focused on endocrine therapy for breast cancer, as discussed in the following sections.

# **NORMAL BREAST**

## **STRUCTURE AND FUNCTION OF BREAST**

### **Structure**

The human breast is a modified apocrine gland and develops as a dermal-derived organ lying within the subcutaneous tissue. The breast rests on the pectoralis muscle and on a thin layer of loose areolar tissue the retromammary space, containing lymphatics and small vessels. The mature breast is composed of three principal tissue types: glandular epithelium, fibrous stroma and fat. The lobule is the basic structural unit of the mammary gland. The number and the size of the lobules vary enormously. In youth, the predominant tissues are epithelium and stroma, which are replaced by fat in older women.

### **Function**

The breast undergoes changes throughout reproductive life. In adolescence, the breast is composed primarily of dense fibrous stroma and scattered ducts lined with epithelium. With puberty there is increased deposition of fat, formation of new ducts and the first appearance of lobular units. This process of growth entails cell division and is under the control of hormones. After the third decade, the ducts and lobules atrophy with shrinkage of the intralobular and interlobular stroma. Menopause results in involution and a generalised decrease in the epithelial elements of the resting breast. These changes include increased fat deposition, diminished connective tissue and the disappearance of lobular units.

## Regulation

Postnatal mammary gland development is highly dependent on the ovarian steroids, oestrogen and progesterone [Woodward TL]. The mammary gland sequentially acquires and cyclically exhibits proliferative responses to oestrogen and progesterone from birth to postmenopause [Fendrick JL]. Oestrogen and progesterone act in an integrative fashion to stimulate normal adult female breast development. Although oestrogen and progesterone are vital to mammary growth, they are ineffective in the absence of anterior pituitary hormones [Fendrick JL]. Oestrogens are considered to play a major role in promoting cell proliferation, while progesterone influences its differentiation [Russo J].

The initiation and progression of breast development involves a coordinated effort of pituitary and ovarian hormones, as well as local mediators [De Bortoli M]. These hormones guide mammary gland development and differentiation by regulating the expression of local growth factors and their receptors at the cell surface [De Bortoli M]. The mammary epithelial proliferation is also probably mediated through stromal-derived factors [Xie J]. Walden et al demonstrated that GH-stimulated production of IGF-1 mRNA in the mammary gland itself, suggesting that IGF-1 production in the stromal compartment of the mammary gland acts locally to promote breast development. Stromal cells appear to influence behaviour by the secretion of growth factors and by altering the composition of the extracellular matrix (ECM) in which epithelial cells reside. The regulation and action of autocrine and paracrine growth factors (including the epidermal, insulin-like, hepatocyte and fibroblast growth factor families) are

probably involved in ovarian steroid mediated epithelial stromal interactions [Woodward TL, 2000]. Hansen et al have suggested that the coordinated regulation of ovarian hormone responsiveness and extracellular matrix / integrin expression may be critical to normal mammary gland development and breast cancer growth and progression. Over the last decade, studies have revealed that basement membrane, extracellular matrix, proteins and their cellular receptors regulate cell proliferation and differentiation and are regulated by complex epithelial stromal interactions regulated by ovarian steroids [Haslam SZ]. Thus, development and regulation of breast is critically dependent upon the nuclear steroid hormones, a number of peptide hormones and various growth factors.

# **STEROID HORMONES**

## **Classes**

Steroid hormones comprise families of structurally related compounds, which include progesterones, corticosteroids, androgens and oestrogens. All share common precursor molecule cholesterol from which they are derived.

## **Mode of action**

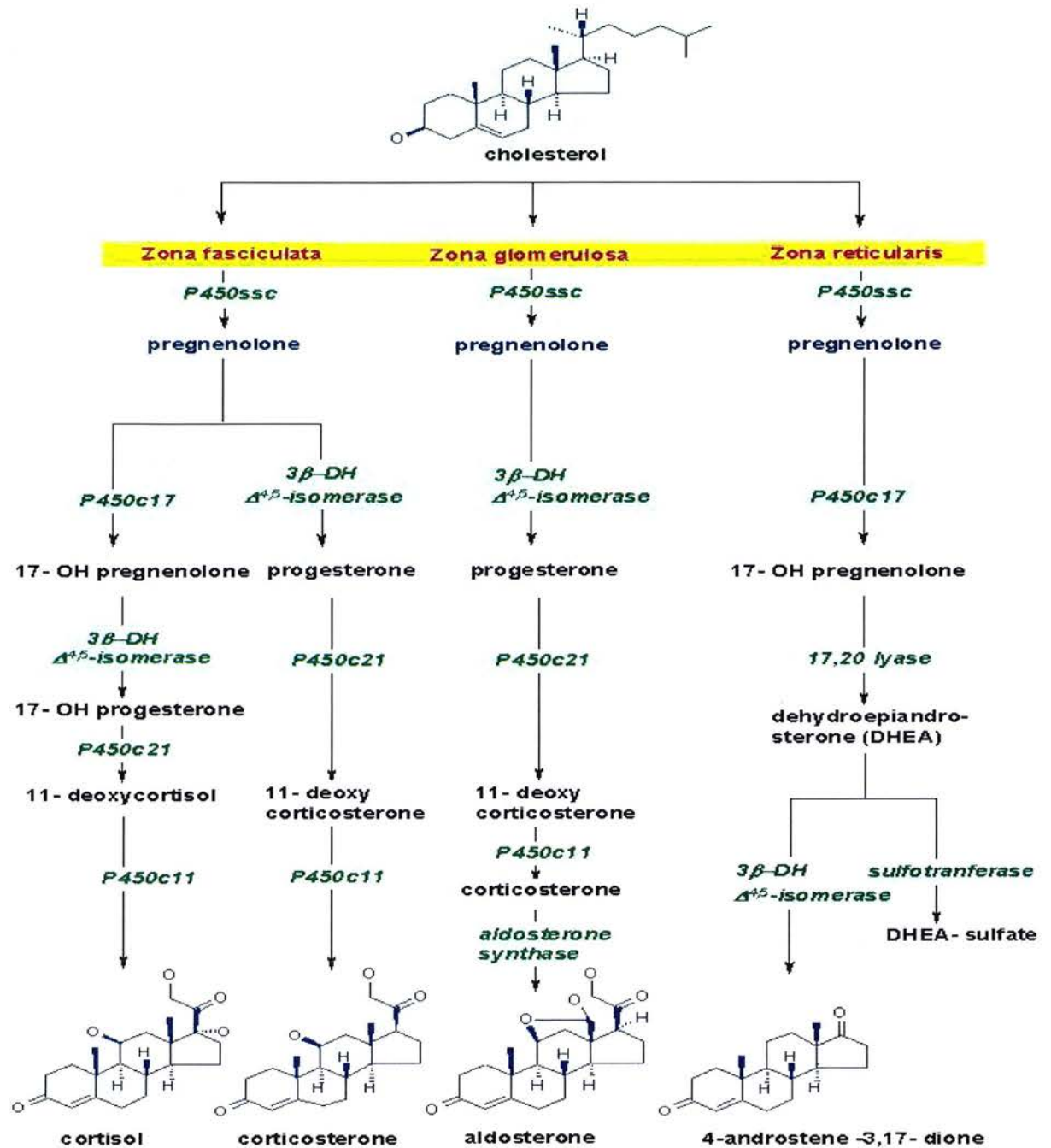
Biologically active steroids are classically produced and secreted from endocrine organs such as ovary, testis and adrenal cortex. They are then transported through circulation and act on target tissues in which their specific nuclear receptors are present [Miller WR, 1996]. The biological features of steroid dependent target tissues are influenced by serum or plasma concentration of the hormones [Suzuki T, 2005]. The biochemical actions of steroid hormones are diverse. In the target tissues steroids may increase the rate of messenger ribonucleic acid (mRNA) synthesis and regulate mRNA stability, increase nucleotide incorporation into deoxyribonucleic acid (DNA), DNA synthesis and cell numbers, enhance the synthesis of specific proteins, member trafficking and mobilisation of lipids. Steroids have pleiotropic effect and are able to induce synthesis and secretion of peptide growth factors, which have autocrine and paracrine influences including proliferative responses [Sasano H, 2007]. Consequently steroids are able to regulate tissue growth, differentiation and metabolic activity. Action of steroid hormones is mediated through receptors, which are essentially nuclear transcription factors [Baniahmad A]. Binding of ligand to receptor causes disassociation of the receptor from chaperone proteins, conformational changes and increase phosphorylation

of the receptor molecule. This, results in transactivation of the receptor promoting transcriptional events necessary for transcription and as a result new species of mRNA appear which may be specific for the interaction of steroids with receptors.

## **Synthesis**

Cholesterol has a phenanthrene structure composed of one five membered and three six membered ring. Steroid biosynthesis comprises a series of degradative steps (**fig 1a**) whereby, C27 sterol is converted [i] to corticoids by partial removal of the side-chain of the D-ring into progestins and its hydroxylation [ii] to androgens by total removal of the D-ring side-chain [iii] to oestrogens by removal of the side chain between A and B rings. Only the synthesis and relevance of steroids to breast are discussed in this chapter.

Fig 1a: Biosynthesis of steroid hormones from cholesterol



## Androgens

The ovary secretes a variety of C<sub>19</sub> steroids, including dehydroepiandrosterone (DHEA), androstenedione, testosterone, and dihydrotestosterone [Labrie F, 2003]. They are produced by the thecal cells and to a lesser degree by the ovarian stroma. The major C<sub>19</sub> steroid androstenedione is partly secreted directly into plasma, while the remainder is converted to oestrogen by the granulosa cells [Suzuki T, 2007]. Androstenedione can be converted to oestrogen or testosterone in the ovary and in extraglandular tissues.

## Oestrogens

The naturally occurring oestrogens are C<sub>18</sub> steroids characterised by the presence of an aromatic A- ring, a phenolic hydroxyl group at C-3 and either a hydroxyl group (oestradiol) or a ketone group (oestrone). In women oestrogens originates from different sources; the ovaries and extragonadal sites are its main sources in the pre and postmenopausal periods respectively. The most potent oestrogen secreted by the ovary is 17 beta oestradiol [Chappel SC]. Oestriol (16-hydroxyoestradiol) is produced by the metabolism of oestrone and oestradiol and is the most abundant oestrogen in urine. Oestrone sulfate is formed by peripheral conversion of oestradiol and oestrone and it is the most abundant oestrogen in blood, though not physiologically active [Vignon F]. Oestrone is mainly derived from extraglandular conversion of androstenedione in peripheral tissues [Lipsett M].

The conversion of androgen to oestrogen in postmenopausal women has shown to occur principally in peripheral tissues including skin, muscle, fat and bone [Longcope C]. The



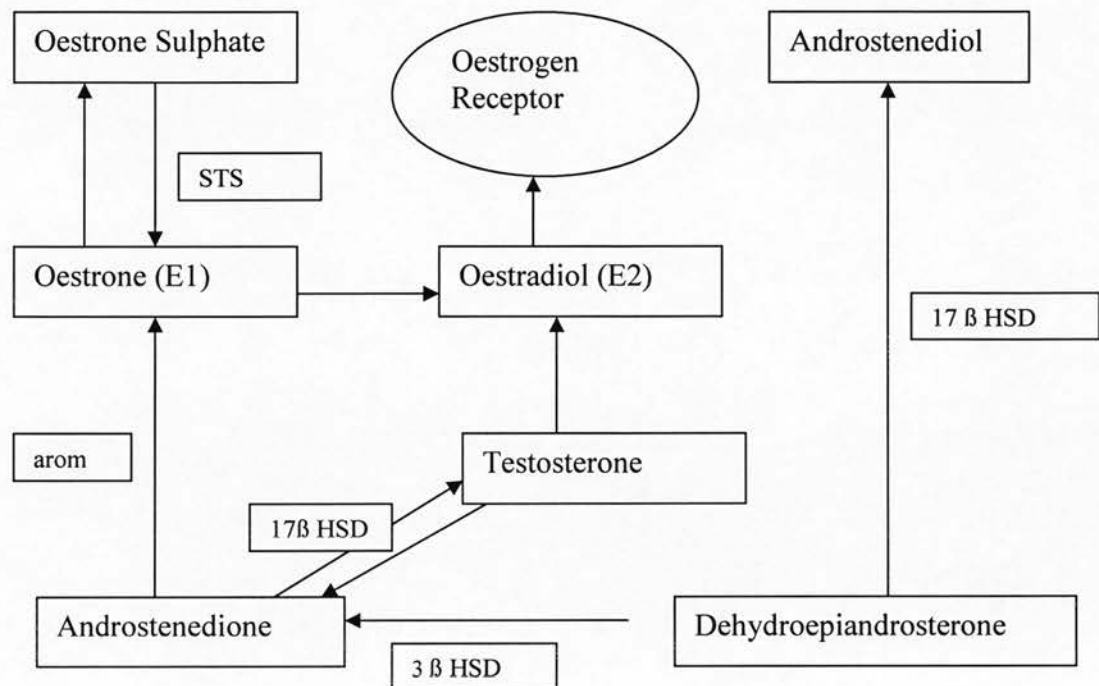
conversion is catalysed, by the aromatase enzyme complex. C19 steroids produced by the adrenal cortex include androstenedione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS). DHEA must be first converted to androstenedione before aromatisation. Another major step is reduction of the 17 keto group to 17 hydroxyl catalysed by 17  $\beta$  hydroxysteroid type I which is essential for the formation of oestrogen (oestradiol).

The extragonadal sites of oestrogen biosynthesis differ from the ovaries in many ways. Although they have the capacity to convert C19 steroids to C18 steroids, unlike the ovaries they lack the ability to synthesise C19 precursors. They are therefore dependent on circulating C19 steroid precursors for oestrogen biosynthesis. Oestrogens synthesised extragonadally are probably only biologically active in local tissues in either an intracrine or a paracrine fashion [Labrie F]. The total amount of oestrogen synthesised by the extragonadal sites may be small but may exert significant biological influence locally.

The major routes of metabolism of oestrogen are [i] interconversion of oestrone and oestradiol [ii] the formation of catechol oestrogen by 2 hydroxylation and [iii] conjugation of oestrogen with sulfate or glucuronic acid. Oestrogen is metabolised almost exclusively by oxidation, in both men and women [Lippert TH]. The first stage involves transformation of oestradiol into oestrone by oxidation at the C17 position, a process that is reversible. Further metabolism, from oestrone takes place in two different ways - by hydroxylation at either the A or D ring. The products of both pathways are catalysed by different enzyme systems. Metabolism of A-ring results in the metabolites 2 and 4 hydroxyoestrone and metabolism of the D ring leads to 16 hydroxyoestrone and

oestriol. In men and postmenopausal women, oestrogen is formed predominantly via aromatisation of adrenal steroids resulting in oestrone and metabolism starts from oestrone [Siiteri PK, 1973].

**Fig 1b: Origin of oestrogen in breast cancer**



HSD -Hydroxy steroid dehydrogenase, STS- Sulfotransferase

## **Intratumoral production of oestrogens in breast**

Studies, have suggested that local formation of oestrogens in the breast tumors is more important than circulating oestrogen in plasma for the growth and survival of oestrogen-dependent breast cancer in post-menopausal women [Foster PA; Russo J; Bulun SE, 2008]. The rationale for the importance of local formation of oestrogens is based on the following; (i) Oestradiol (E2) levels in breast tumors are equivalent to those of pre-menopausal patients, although plasma E2 levels are 50-fold lower after menopause. E2 concentrations in breast tumors of post-menopausal women are 10-40 times higher than serum level [Nakata T, 2003] (ii) breast tissues are able to concentrate oestrogen from the circulation against a gradient [Miller WR, 1997] and (iii) mammary adipose tissue and breast cancers are capable of oestrogen biosynthesis [Varela RM].

Local potential for oestrogen production within the breast is relevant to the natural history of breast cancer. Both normal and diseased breast can produce steroid hormones, which can influence the growth and development of breast tissues [Varela RM]. Perel and Miller et al independently demonstrated the ability of human breast and its neoplasms to produce  $17\beta$  oestradiol (invitro) [1982].

Biosynthesis of oestrogens in breast tumors tissues occurs via two major different routes, (**Fig 1b**) one is aromatase pathway and another is steroid-sulfatase (STS) pathway. Intratumoral aromatase plays as important role converting serum androgens to oestrogens in situ and serves as a major source of oestrogen. However, other enzymes such as the 17 beta-hydroxysteroid dehydrogenase isozymes (17beta-HSD), oestrogen

sulfatase (STS) and oestrogen sulfotransferase may also play pivotal roles in intratumoral oestrogen production [Sasano H, 2006].

High concentrations of circulating inactive steroids, such as androstenedione and oestrone sulfate, are considered major precursor substrates of local oestrogen production [Suzuki T, 2006]. The relative contribution of uptake into the breast and local biosynthesis of endogenous oestrogens within breast tumours can be assessed from infusion studies [Reed MJ, 1991]. These suggest substantial inter-subject variation; in some breasts, oestrogen levels are largely accounted for by uptake, whereas in others most oestrogen appears to be derived from local intratumoural synthesis [Bulun SE, 1994; Miller WR, 2006]. Furthermore, tumours with highest levels of oestrogen are invariably those with the greatest in situ synthesis [Reed MJ, 1991; James VHT, 1989]. If intratumoural aromatase activity is the source of oestrogen, which maintains malignant growth, treatment with drugs, which inhibit that activity, would be particularly effective in these tumours.

## OESTROGEN AND BREAST CANCER

Breast cancer risk is related to the cumulative exposure of the normal breast epithelium to the sex hormones, mainly oestrogen. It has been well documented to play a central role in the aetiology of breast cancer [Travis RC].

Several endogenous and exogenous variables, which alter oestrogen status, affect the risk of breast cancer [Henderson BE, 1981, 1984]. The most consistently documented hormonal risk factors are early menarche, late menopause and at first full-term pregnancy [Brinton LA, 1988; Henderson BE, 1985; Trichopoulos D]. The intense differentiation of the terminal duct lobular unit associated with each full term pregnancy and release of various hormones, autocrine and paracrine growth factors during lactation may explain the protective effects of early age at first full term pregnancy, parity and lactation of breast cancer risk [Okobia MN, 2005]. Observations suggest that regular ovulatory cycles increase the risk of developing breast cancer [Henderson BE, 1985]. Women with early menarche and rapid establishment of regular cycles had an almost four-fold increased risk of breast cancer compared to women with late menarche and long duration of irregular cycles [Vihko RK]. Rate of breast cancer increases rapidly in the premenopausal years, but slows sharply at the time of menopause, when endogenous hormone levels decline rapidly [Sasco AJ]. The rate of breast cancer in the postmenopausal period is only about a sixth of the rate in the premenopausal period. This age incidence curve appears to be shaped in a major way by the effects of ovarian activity [Henderson BE, 1993].

Hormone replacement therapy and oral contraceptives, the exogenous sources of oestrogens, are also potential contributors to breast cancer risk [Collaborative Group, 1997]. The Million Women Study (MWS) and the Women's Health Initiative (WHI) study demonstrated an increase in breast cancer risk in women on hormone replacement therapy (HRT) in comparison with control groups. In the Million women study current users of HRT were associated with an increased risk of incidents (RR 1.66,  $p < 0.0001$ ) and fatal breast cancer (RR 1.22,  $p = 0.05$ ). Incidence was significantly increased for current users of preparations containing oestrogen only (RR 1.30,  $p < 0.0001$ ), oestrogen progesterone (RR 2.00,  $p < 0.0001$ ), but the magnitude of the associated risk was substantially greater for oestrogen- progesterone than other types of HRT ( $p < 0.0001$ ). Results varied little between specific oestrogens and progesterone or their doses; or between continuous and sequential regimens. In current users of each type of HRT, the risk of breast cancer increased with increasing total duration of use. 10 years use of HRT is estimated to result in five additional breast cancers per 1000 users of oestrogen only preparations and 19 additional cancers per 1000 users of oestrogen-progestrone combinations. In the WHI trial women randomised to oestrogen and progesterone, had an elevated risk of breast cancer (RR = 1.29) after 5 years of follow-up.

The relationship between weight and breast cancer risk is critically dependent on age. In postmenopausal women, a 10 kg increment in body weight results in an approximately 80% increase in the risk of breast cancer [den Tonkelaar I]. This is due to the higher circulating oestrogen level from conversion of the adrenal androstenedione to oestrone by enzymes present in body fat.

In premenopausal women, the relationship between weight and breast cancer risk is less clear-cut, but may be inverse [Waard F]. Nutrition probably influences breast cancer occurrence by modifying age at menarche and body weight. Howe et al found that the breast cancer risk of postmenopausal women was positively associated with both total fat and saturated fat intake [Brinton LA, 1995]. However, analysis of cohort studies has found little or no difference in breast cancer-risk and intake of fat [Hunter DJ].

A large and compelling body of epidemiological data, animal studies and clinical trials implicate oestrogen in the aetiology of breast cancer [Henderson BE, 1988]. It is well known that synthesis and metabolism of oestrogen takes place in both tumour and surrounding tissues [Santen RJ, 1999]. Administration of steroids in patients with breast cancer can increase tumour cell proliferation [Dao TL]. Experimentally this is manifested by the proliferative effect on cultured breast tumour cell lines and development of mammary tumours in rats exposed to high doses of oestrogen [Rudali G; Lippman ME, 1975]. Higher plasma oestradiol levels have been reported several years before diagnosis in women with breast disease compared with matched controls [Toniolo PG]. An inverse relationship between plasma oestrogen and disease free interval has been demonstrated in premenopausal breast cancer patients after primary treatment [Lonning PE, 1996].

The most convincing data that oestrogens play a major role in the natural history of breast cancer are from treatment aimed at inhibiting oestrogen production by ovarian ablation or administration of antioestrogens [Veronesi U, 1975]. Artificial menopause induced by either bilateral oophorectomy or pelvic irradiation, also markedly reduces



breast cancer; this effect appears to be slightly greater than that of natural menopause [Parazzini F].

Endocrine therapy has shown that surgical, radiological or medical oophorectomy causes regression of about 30% of breast cancers in premenopausal women [Ward HWC]. Remission is noted in a similar proportion of postmenopausal women treated by adrenalectomy, hypophysectomy, antioestrogens and aromatase inhibitors.

Evidence suggests that the genotoxic transformation can be initiated by binding of the electrophilic metabolite of oestradiol or oestrone to deoxyribonucleic acid (DNA), initiating the critical step in the pathway leading to tumour formation [Cavalieri EL]. Studies indicate that metabolites of both oestradiol metabolism pathways can potentiate carcinogenesis [Theodor HL]. Oestrogens are considered typical tumour promoters; due to their oestrogen-receptor mediated mitogenic activity that increases the probability of spontaneous mutations [Emons G]. Oestrogens may also exert paracrine effects on tumour development and growth by increasing the expression and activity of other regulatory proteins including transforming growth factor-alpha, cyclin-dependent kinases and epidermal growth factor [Fernandez PL; Yee D].

## **Endocrine Sensitivity**

### **Oestrogen mediated effects**

Normal breast growth and development are regulated by the complex interaction of hormones and growth factors [Dickson RB, 1988; Osborne CK, 1990]. Abrogation's in hormonal regulation (endocrine, paracrine, autocrine and intracrine) contribute to breast tumourigenesis by promoting inappropriate growth and survival of cells. Studies indicate that breast cancer cells under oestrogenic control synthesise and secrete their own growth factors, which may auto stimulate themselves or adjacent stromal tissues through autocrine or paracrine mechanism [Lippman ME, 1987]. Therefore, it is important to understand the direct and indirect effects of oestrogen on breast, as it influences the natural history of breast cancer.

### **Mechanisms of oestrogen action**

The major effects of oestrogen are probably mediated directly on tumour cells through the oestrogen receptors, which programme genomic responses [Yamamoto KR]. Oestrogen can also indirectly affect the growth of breast cancer by influencing polypeptide hormones and secretion of mitogenic factors [Freiss G]. It is unclear whether receptor-genome interaction directly modifies cellular proliferation or if growth effects are mediated via secondary response modifiers. However, in oestrogen-sensitive cell lines of breast cancer, oestrogen may (i) induce the secretion of mitogenic factors and (ii) stimulate the production of

proteases, which may degrade extracellular matrix and thereby accelerate tumour invasion and metastasis.

## **Hormone receptors**

Reproductive and hormone sensitive tissues possess high-affinity protein receptors for oestrogen and progesterones. Receptors may be expressed in normal and tumour tissue of mammary origin [McGuire WL, 1975]. Isolation of the steroid receptors has led to understanding of the molecular mechanism of action of the steroid hormones. Hormone receptor proteins are activated when occupied by their specific hormone ligand receptor, leading to induction of numerous cellular genes, including those that may encode critical enzymes and secrete peptide growth factors [McInerney EM]. Intracellular binding of the steroid-receptor complex to DNA at different promoter regions of numerous oestrogen-responsive elements results in several biologic effects including stimulation of mammary gland growth [Kelloff GJ]. Therefore, it is likely that the proliferative effect of steroids on tumour cells is also mediated through the receptor proteins.

## **Oestrogen receptors**

Oestrogen receptors (ER) have two isoforms: the initially cloned oestrogen receptor alpha and the more recently cloned oestrogen receptor beta [Mosselman S]. Oestrogen stimulates cell growth by binding to the oestrogen receptor, which causes oestrogen receptor homodimer formation [Read LD]. This receptor-ligand complex binds to specific DNA sites called oestrogen-responsive elements and serves as a transcription factor, activating oestrogen-responsive genes that regulate the cell growth [Green S].

Thus oestrogen signal transduction plays an important role in both normal and neoplastic mammary tissue [Vorherr H]. However, perturbations of oestrogen receptor signal transduction are thought to contribute to tumour progression and the eventual development of a hormone-independent and more aggressive phenotype [Schmitt FC].

## **Progesterone receptor**

Progesterone receptor may be an indicator of a functional oestrogen receptor, which may explain why progesterone receptor-positive breast cancers respond to hormonal treatment, despite very low or absent oestrogen receptor [Horowitz KB]. The predictive value of PR has long been attributed to the dependence of PR expression on ER activity, with the absence of PR reflecting a nonfunctional ER and resistance to hormonal therapy [Wilson CA]. However, recent clinical and laboratory evidence suggests that ER positive/PR negative breast cancers may be specifically resistant to SERMs, where as they may be less resistant to oestrogen withdrawal therapy with aromatase inhibitors, which is a result inconsistent with the nonfunctional ER theory [Arpino G; Howell A, 2005]. Novel alternative molecular mechanisms potentially explaining SERM resistance in ER positive/PR negative tumors has been suggested by experimental indications that growth factors may down regulate PR levels [Cui X, 2005]. Thus, the absence of PR may not simply indicate a lack of ER activity, but rather may reflect hyperactive cross talk between ER and growth factor signaling pathways that down regulate PR even as they activate other ER functions.

## **Growth factors**

Tissue regulation is modulated in a very complex fashion by locally acting polypeptide hormones (growth factors). Growth factors include epidermal growth factor (EGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF) and transforming growth factors (TGF family). The EGF family includes four transmembrane tyrosine kinase receptors, HER 1-4 and several growth factors including EGF and TGF- $\alpha$ . Multiple aspects of breast biology, including survival, proliferation and differentiation are modulated by the EGF family through complex receptor heterodimerization pattern [Earp HS]. TGF- $\alpha$  and related growth factors may also mediate the stromal epithelial interactions involved in the indirect growth response of normal mammary epithelial cells (MECs) to oestrogen. Thus antibodies against TGF- $\alpha$  or its receptor (EGFR) can block oestrogen induced growth [Dickson RB, 1998]. Studies suggest that oestrogen receptor negative breast cancer cells are more sensitive to TGF- $\beta$  [Arteaga CL]. The malignant potential of breast cancer is likely to depend on the balance between the stimulators and inhibitors of growth produced by the tumours. TGF- $\beta$  may inhibit the proliferation of epithelial tissues and stimulate the proliferation of stromal tissues via interdependent serine-threonine kinase receptors [Relf M]. Insulin-like growth factor 1 plays a central role in normal mammary gland development. Insulin-like growth factors 1 and 2 are both potent mitogens for breast cancer cells invitro [Surmacz E]. Expression of several fibroblast growth factor (FGF) ligands and receptors has been observed in normal and malignant breast tissue [Kern FG]. Fibroblast growth factors (FGF) have pleiotrophic effects on breast tumour cells including increased metastatic ability (invivo)

and angiogenesis [Lee AV]. The epithelial and/or stromal cells within the tumour also secrete proteases, such as the cathepsins, stromelysin, gelatinases or urokinase plasminogen activator, which may contribute to tumour invasiveness and metastatic potential [Noel A; Thomssen C]. In oestrogen receptor positive breast cancer cells, expression and secretion of certain autocrine growth factors, such as TGF- $\alpha$  and IGF-2, are stimulated by oestrogen and inhibited by antioestrogens while oestrogen receptor negative breast cancer cells are autonomous [Lippman ME, 1987]. Thus changes in the expression of these secreted factors can mediate the growth effects of oestrogen and antioestrogen. If the actions of oestrogen are mediated by growth factors, oestrogenic responses should be mimicked by growth factors. Conversely, anti-growth factor strategies should cause regression of oestrogen-dependent tumours. Experimental systems do not completely support these concepts. The full growth-promoting effects of oestrogen seem to require other agents besides secreted growth factors. The sequelae from the observations are that therapies directed against oestrogen-induced growth factors are unlikely to be as effective in causing tumour regression as antioestrogen and conversely, successful antioestrogen therapy is not simply mediated via antagonism of growth factor production, but embodies full panoply of actions.

## ENDOCRINE DEPRIVATION

Tumours of target organs for steroid hormones, most notably breast cancer, often need oestrogen for their continued growth and will regress if deprived of such hormones [Bulbrook RD; Miller WR, 1996]. In contrast to prostatic cancer, in which the majority of patients respond to endocrine therapy, only a third of the patients with advanced breast cancer respond to hormonal manipulation [Masamura S]. Endocrine blockade aims to deprive hormone responsive cancer cells of mitogenic stimulation by oestrogen. Before the menopause the ovary is the principle source of oestrogen; it explains the beneficial effects of surgical or radiation induced oophorectomy in premenopausal women with breast cancer [Clarke MJ]. The glandular production of oestrogen becomes negligible after menopause and biosynthesis in peripheral sites becomes physiologically important [Poortman J]. Oestrogen deprivation therapies aim to destroy hormone producing tissues either by surgical intervention or by the use of drugs, which inhibit the enzymes involved in oestrogen biosynthesis at the oestrogen receptor. Endocrine deprivation may also induce growth inhibitory factors or reduce the production of growth stimulatory factors [Hulka BS].

Endocrine therapies can be classified as ablative, additive, inhibitive and competitive therapy. Most of them affect tumour growth, either by inhibiting the synthesis of oestrogen or its action at the level of the tumour cell [Goldhirsch A]. Other postulated mechanisms include direct cytotoxicity, effect on autocrine growth factors, apoptosis and cell cycle arrest. The choice of endocrine therapy is influenced by the menopausal status. In premenopausal women, oestrogen synthesis is reduced by ovarian ablation

with surgery, radiotherapy, or luteinizing hormone-releasing hormone (LHRH) agonists [Gradishar WJ]. Aromatase inhibitors are largely ineffective in this group as peripheral suppression is overcome by the intact hypothalamic-pituitary-ovarian axis [Santen RJ, 1980]. In postmenopausal women, antioestrogens, aromatase inhibitors and progestins are effective [Miller WR, 1990]. **Table – 1** considers different strategies employed for oestrogen deprivation.



**Table-1: Oestrogen deprivation strategies**

Primary Effect	Strategy
Decrease oestrogen production	<p><b>Irreversible surgical ablation:</b> oophorectomy, hypophysectomy, adrenalectomy</p> <p><b>Reversible medical ablation:</b> luteinizing hormone-releasing hormone agonists</p> <p><b>Inhibitive strategies:</b> selective aromatase inhibitors and non selective aromatase inhibitors</p>
Antagonise oestrogen effects	<p>Competitive: antioestrogens</p> <p>Mechanism unclear: progestins, androgens</p>

## **Oestrogen deprivation and transition to hormone independence**

Antihormones continue to be a mainstay in the management of breast cancer disease, promoting worthwhile tumour remissions and significant survival benefits in many ER positive patients [Santen RJ, 2004]. Some of these agents act by competing with oestrogen for binding to its target receptor in breast cancer cells, exemplified by partial antioestrogens (Tamoxifen) and also pure antioestrogens (Faslodex) that additionally reduce ER level. Other agents, notably aromatase inhibitors, act to severely deplete the oestrogenic environment. The efficacy of all antihormones present to date is compromised not only by a proportion of ER positive patients exhibiting de novo antihormone resistance, but also by the many more who acquire resistance following an initial therapeutic response [Gee JMW; Johnston SR].

Deprivation of oestrogen causes breast tumors in women to adapt and develop enhanced sensitivity to steroids [Santen RJ, 2008]. Following successful endocrine treatment, most hormone-dependent tumours may become independent and resume growth. It is therefore important to define mechanisms by which autonomy occurs and to characterise the events, which may follow oestrogen deprivation. In oestrogen, deprived environment the endocrine system may compensate by producing oestrogen from alternate sources [Miller WR, 1999]. But in most patients, relapse after successful endocrine treatment is not associated with an increase in oestrogen and when raised levels occur they seem to be the consequence rather than the cause of progressive disease.

The concept that hormone independence results from constitutive production of the growth factors, which are normally induced by oestrogen, has received considerable attention. Studies in which breast cancer cells have been engineered to overproduce growth factors in the absence of oestrogen failed to demonstrate that this could lead to an oestrogen-independent phenotype [Clarke R]. Preliminary results in primary breast cancers did not support the theory that oestrogen-independent tumours are autonomous on account of enhanced growth factor synthesis [King RJ]. There is a growing body of literature suggesting that cross-talk between growth factors and oestrogen receptors may contribute to endocrine resistance [Massarweh S]. Based on studies primarily with tamoxifen (but now emerging with oestrogen deprivation), altered growth factor receptor signalling and its cross-talk with the ER pathway has been heavily implicated as a resistance mechanism [Hutcheson IR]. Of particular interest is the recent finding with respect to human epidermal growth factor receptor 2 over expression, suggesting this may reduce responsiveness to tamoxifen but not to aromatase inhibition [Jones HE].

During oestrogen deprivation renewed growth may likely result from adaptive changes within the tumour either from outgrowth of oestrogen-resistant cell clones (which were present from the outset of therapy) or from cell genetic changes resulting in autonomous phenotype [Powers MV]. These phenomena need not be mutually exclusive and there is supportive evidence for both. Firstly, cellular heterogeneity exists within tumours with regard to hormone sensitivity and successful hormone therapy can produce selective cell kill. Secondly, adaptive changes occur in oestrogen-dependent breast cancer cells maintained in a steroid

free environment; in time, an oestrogen-resistant phenotype develops [Darbre PD]. Loss of sensitivity to oestrogen can also occur without concomitant loss of oestrogen receptors or oestrogen-inducible molecular markers. The exact molecular changes essential for autonomy have not been determined although different patterns of mRNA expression may be observed in oestrogen-dependent and independent cells. The acquisition or increased capacity of tumour cells to synthesise oestrogen would theoretically confer independence from an external source of oestrogen. In addition, recent findings by Modlich et al suggest that it is important to assess changes in gene expression that might occur after the initiation of therapy. Thus, prolonged endocrine therapy can be associated with an acquired increase in peptide growth factor signaling (EGFR, HER2), together with cross talk, activation of ER dependent gene transcription and cell growth. all of which may lead to endocrine resistance [Johnston SR]. All in all, a better understanding of the multidirectional cross-talk between growth factor pathways, the oestrogen receptors (both at the genomic and nongenomic level), and oestrogen receptor cofactors is vital to overcome endocrine resistance [Santen RJ, 2006].

## ENDOCRINE THERAPY

### Background to endocrine therapy

Hormonal therapy for breast cancer has changed radically from ablative surgical procedures to medical therapy. In 1886, Beatson first demonstrated that bilateral oophorectomy leads to regression of metastatic breast cancer. Surgically induced menopause became the first effective means to control advanced breast cancer, producing a beneficial regression in 25 - 40% of premenopausal patients [Beatson G, 1901]. In 1922, DeCourmellers administered radiotherapy to the ovaries; its antitumour effect was similar to surgical oophorectomy, except for the delay in tumour regression. In 1952, Huggins demonstrated the effectiveness of adrenalectomy in the treatment of postmenopausal metastatic breast cancer. In 1953, Luft proved that hypophysectomy which deprives pituitary polypeptide hormones, caused palliative remission in upto 40% of patients. These procedures have largely been abandoned due to their associated morbidity.

A greater understanding of oestrogen action on tumour growth evolved with time and new medical treatments were developed (**table-2**). Over the last few decades, antioestrogen therapy has almost replaced endocrine organ ablation. The first experience of using, androgens, oestrogen and progestins as additive therapies were reported in the 1950s [Ulrich P, Stoll BA]. Tamoxifen, an oestrogen agonist-antagonist was introduced in 1970 and used in treatment of oestrogen-sensitive breast cancer [Cole MP]. In 1970s aminoglutethimide, an aromatase inhibitor, replaced adrenalectomy in the treatment of advanced breast cancer [Griffiths CT]. In the last decade, aromatase inhibitors of the

second generation (fadrozole, formestane) and third generation (anastrozole, letrozole, exemestane) and new anti-oestrogens (toremifene, raloxifene, droloxifene) have been developed. Newer generation aromatase inhibitors are far more enzyme specific with minimal side effects and have almost replaced tamoxifen in the treatment of hormone sensitive breast cancer.

**Table – 2: Modalities of hormonal therapy**

<b>Year</b>	<b>Therapy</b>	<b>Pioneer</b>
1886	Oophorectomy	Beatson
1939	Androgens	Ulrich
1951	Progestins	Esher
1952	Pituitary irradiation	Douglas
1953	Pituitary removal	Luft
1971	Antioestrogen	Cole
1973	Aromatase inhibitors	Griffiths
1982	Luteinizing hormone releasing hormone agonists	Klijn
1987	Antiproggestins	Romieu
1993	Pure antioestrogen	Howell

## METHODS OF ENDOCRINE DEPRIVATION

All endocrine therapies aim to reduce oestrogen dependent tumour growth. Surgical procedures such as oophorectomy ablate oestrogen synthesis by the ovary [Veronesi U, 1975]. Antioestrogens act at the cellular level by interfering with the action of oestrogen on the hormone receptors, while aromatase inhibitors inhibit the peripheral conversion of steroid precursors into oestrogen [Jordan VC, 1997]. The mechanism of action of additive therapies such as progestins, androgens and oestrogens are not clearly understood. **Table - 3** illustrates the different mechanisms of action.



**Table-3: Mechanism of action of endocrine therapies**

<b>THERAPY</b>	<b>MECHANISM OF ACTION</b>
<b>Oophorectomy</b>	Ablation of oestrogen synthesis in premenopausal women
<b>Adrenalectomy</b>	Decreased androgens leading to decrease in their aromatisation to oestrogen
<b>Hypophysectomy</b>	Decreased FSH, LH, and corticotropin leading to decreased oestrogen synthesis
<b>Ovarian radiation</b>	Primary site of oestrogen synthesis in premenopausal women removed; decreased oestrogen synthesis
<b>Antioestrogens</b>	Competition with oestrogen for binding to oestrogen receptor
<b>Aromatase inhibitors</b>	Aromatisation of androgens into oestrogen inhibited in postmenopausal women
<b>LHRH agonists</b>	Gonadotropin stimulation of hypothalamus blocked, thereby decreasing release of FSH/LH; oestrogen synthesis decreased in premenopausal women
<b>Progestins</b>	Unclear

FSH-follicle stimulating hormone; LH-luteinizing hormone; LHRH-luteinizing hormone releasing hormone

## **Predictive markers to endocrine therapy**

Oestrogen receptor (ER) has been studied in clinical breast cancer for more than 20 years [Love RR, 1990] and is widely used to select patients for adjuvant hormonal therapy [Allred DC, 1998; Harvey DC, 1999]. It is clear that ER status (ER positive or negative) remains the most logical and reliable predictor of response to endocrine therapy in the primary (neoadjuvant), adjuvant and metastatic settings [Pritchard KI.]. Approximately 50%–60% of women with ER-positive breast cancer benefit from endocrine therapy [EBCTCG, 1992]. Research carried out in the early 1970s showed that the ER protein was present in 50–70% of invasive breast cancers [Jordan VC]. Based on a pooled analysis of 400 patients with advanced breast cancer, McGuire et al showed that 50–60% of women possessing ER positive tumors responded to endocrine therapy. In contrast, only 5–10% of ER-negative tumors regressed with this treatment. It was later shown that 70–80% of breast cancers containing both ER and PR regressed with hormone therapy [McGuire WL, 1980]. Bardou et al demonstrated that the combined measurement of ER and PR is superior to ER alone in predicting benefit from adjuvant hormone therapy. Although response rates progressively decline, they remain in the 20–40% range [Cheung KL, 1997]. Irrespective of the type of hormone therapy used, only 30% of unselected patients with metastatic breast cancer respond [Buzdar AU, 1998].

Approximately 50 to 60% of all advanced breast cancer in postmenopausal patients will have hormonally responsive disease [Osborne CK, 1991]. Eventually approximately half of patients with acquired resistance obtain a clinical benefit from other endocrine therapies. The National Institutes of Health panel, the European Group on Tumor

Markers, and the European Society of Mastology have recommended that ER and PR be assayed on all primary breast cancers [Blamey RW, 2002]. Currently, most investigators use immunohistochemistry to measure ER and PR [Duffy MJ, 2005]. Baseline tumor ER expression should be present in at least 10% of infiltrating cancer cells and preferably should score in the 7 to 8 range by the Allred scoring method. The Letrozole P024 study required the ER to be expressed in at least 10% of the malignant infiltrating component of the tumor, but in the IMPACT study, only 1% of the cells were required to express ER. It is very clear that neoadjuvant endocrine therapy response rates are closely related to oestrogen-receptor expression [Ellis MJ, 2003].

70% of tumors that have ER or PR do regress in response to these preoperative hormone therapies [Litherland S]. Although 60-70% of patients with ER positive tumours respond, only 30-40% sustains response beyond 2 years [Pritchard KI]. In patients who develop recurrent metastatic disease, it has been a concern as to whether hormone sensitivity due to the presence of ER in the original primary tumor is lost over time. This could account for lower response rates seen with tamoxifen in advanced metastatic breast cancer compared with recent studies when given as primary medical therapy, as it is recognized that 30-40% of metastases from original ER-positive primary breast cancer fail to respond to endocrine therapy [Duffy MJ, 2005].

Expression of ER $\alpha$  is the main predictor of response to endocrine therapy, lack of expression of ER is clearly the main mechanism of de novo resistance to treatment with hormonal agents [Weippl SK, 2005]. Because the presence of ER does not guarantee response, optimal management with endocrine therapy requires the identification of further markers by which to subdivide ER-rich tumours into responders and non

responders [Speirs V, 2004]. Other markers may only be predictive effects for certain types of endocrine therapy. Thus in the P 024 study in which patients were randomised to receive either tamoxifen or letrozole, high expression of epidermal growth factor receptor and c-erbB2 was associated with a decreased likelihood of the response to tamoxifen but an increase of the response rate to letrozole. In results from Edinburgh, in which patients were treated with a third-generation inhibitor (anastrozole, letrozole or exemestane) over expression of c-erbB2 did not appear to be associated with resistance to any of the aromatase inhibitors [Dixon JM, 2005]. Overall, response rates were similar irrespective of c-erbB2 expression. High expression of c-erbB2 did not reduce tumour responses to neoadjuvant treatment with aromatase inhibitors, but was associated with high tumour proliferation before and during treatment. It remains to be determined whether these characteristics may confer subsequent resistance to treatment and early relapse in the adjuvant setting.

Additional factors, which might characterise hormone sensitive versus hormone resistant tumors, might improve on the predictive power of ER status alone for response to endocrine therapy. In particular, the ER phenotype manifested by coexpression of oestrogen-dependent proteins such as pS2 and bcl-2 are thought to represent functional receptor pathway [Horowitz KB]. Arpino et al demonstrated that patients with HER-2 amplification had lower ER levels and were modestly less responsive to tamoxifen, suggesting that molecular events in addition to those involving the ErbB receptors are important in determining the endocrine-resistant phenotype. Significantly lower rate of response to endocrine therapy were seen in patients with high vascular endothelial growth factor (VEGF) levels and high levels of urokinase-type plasminogen activator

[Manders P, 2003, 2004]. Studies using gene expression profiling have demonstrated the distinct genetic patterns of different breast tumors, including at least two subtypes of hormone receptor positive disease, often referred to as luminal A and B [Sorlie T]. More recently, investigators from the NSABP and Genomic Health have demonstrated a 21 gene assay, which can help predict response to endocrine therapy [Paik S]. Specific genes in the expression profile can predict response to preoperative endocrine therapy more accurately and may themselves be candidates as direct targets for future therapies.

## **Antioestrogens**

Antioestrogens fall into at least 2 distinct categories: mixed or partial agonists antagonists such as tamoxifen and pure antagonists such as Fulvestrant. Although both oestrogen and antioestrogen bind within the hormone-binding domain of the oestrogen receptor (ER), the association must differ because oestrogen binding activates transcription, whereas antioestrogens partially or completely fail in this role [Tzukerman M]. The partial agonist-antagonist compounds form complexes with the oestrogen receptor (ER) that bind as dimers to oestrogen-response elements; there they block ligand-dependent transcription activation [Wolf D]. In the case of the more complete antagonists, ER binding to DNA and reduction of the ER content of target cells appear to contribute to, but may not fully explain, the pure antagonist character. The antitumour effect of tamoxifen is mediated primarily through the ER, although there maybe other potential mechanisms of action [Jordan VC, 1997].

## **Tamoxifen**

It was first approved for use in 1977 for the treatment of advanced breast cancer in postmenopausal women. The selective oestrogen receptor modulator, tamoxifen, has been the mainstay endocrine therapy for hormone receptor-breast cancer for several decades. Gradually, tamoxifen is being replaced by the third-generation aromatase inhibitors [Nabholtz JM, 2008].

Data from the Nolvadex Adjuvant Trial Organisation (NATO) study, the Scottish Tamoxifen trial, the Early Breast Cancer Trialists Collaborative Group (EBCTCG) and

the National Surgical Adjuvant Breast and Bowel Project (NSABP) demonstrate the efficacy of tamoxifen in the adjuvant setting. Tamoxifen was considered, as first-line endocrine therapy for all stages of breast cancer and in chemoprevention in high-risk population [Fisher B, 1998]. It was recommended as adjuvant therapy, for early and late stage oestrogen receptor (ER) / progesterone receptor (PR) positive breast cancer and metastatic breast cancer in postmenopausal women and men. The NATO study demonstrated a decrease in the risk of relapse and mortality with tamoxifen as adjuvant treatment in postmenopausal women. The NSABP B-14 [Fisher, 1989] and the EBCTCG meta-analysis update have demonstrated improvement in overall survival in oestrogen receptor-positive women in the premenopausal and the node-negative setting [EBCTCG, 1998]. Regardless of nodal status, tamoxifen has been shown to improve both disease free and overall survival. This benefit is most significant in postmenopausal patients, but benefits were also noted in women less than 50 years of age [EBCTCG, 2005]. Tamoxifen offers the greatest benefit to patients whose tumours are most strongly ER positive [EBCTCG, 1998]. Tamoxifen reduced the risk of recurrence by almost 50% and that of death by 30% when used for 5 years in the adjuvant setting [EBCTCG, 1998]. A secondary benefit of tamoxifen is an approximate 50% decrease in the risk of second primary breast cancers.

The optimal duration of tamoxifen therapy was investigated. The Swedish Breast Cancer Cooperative Group noted that 5 years of adjuvant tamoxifen was more beneficial than 2 years in the treatment of postmenopausal women with oestrogen receptor-positive, breast cancer. The NSABP B-14 trial has shown that the benefit of 5 years of tamoxifen persists through 10 years of follow-up and that no additional benefit is obtained by

continuing tamoxifen for more than 5 years [1996, 2001]. Thus, the standard adjuvant therapy was for five years.

The most frequent adverse reactions to antioestrogens, such as tamoxifen, include hot flushes, nausea and vomiting [Fisher B, 1996]. These may occur to some degree in up to 50% of patients but are rarely sufficiently severe to require discontinuation of therapy. Menstrual irregularities, vaginal bleeding and discharge, pruritis vulvae and skin rashes have occurred less frequently. An increase in risk of tumour flare, thromboembolic events, ocular disturbances, hypercalcaemia and endometrial cancer has also been reported following tamoxifen [van Leeuwen F; Nevasssaari K].

A troubling aspect of tamoxifen use as early adjuvant treatment is the development of *de novo* resistance and thus relapse during the first few years of the planned 5-year course of treatment [Ring A]. However, more than half of hormone-sensitive patients will relapse after 5 years of treatment and two thirds of deaths will occur after completion of the hormonal treatment [Ring A]. This has led to the search for new hormonal agents with the specific goal to improve the therapeutic ratio.

The adjuvant treatment of patients with endocrine-sensitive breast cancer has been dominated for several decades by the gold-standard tamoxifen. In the metastatic setting, superior efficacy over tamoxifen has led to the Aromatase Inhibitors becoming the agents of choice in postmenopausal women with receptor-positive metastatic disease. They now show consistent improvements over tamoxifen in the treatment of early breast cancer, in both the neoadjuvant and adjuvant settings. Thus, the choice of adjuvant endocrine treatment for early breast cancer (EBC) has now evolved from tamoxifen to the AIs. Five years of tamoxifen, the standard adjuvant, endocrine therapy, is now



known to be inferior to treatment with the third-generation aromatase inhibitors (AIs), which are approved for treatment in a variety of adjuvant settings.

## **Selective oestrogen receptor modulators**

The selective estrogen receptor modulators (SERMs) are molecules that block oestrogen action in breast cancer but can still potentially maintain the beneficial effects of oestrogen in other tissues, such as bone and cardiovascular system [Jordon VC, 2008]. Tamoxifen, the prototypical drug of this class has been used extensively for the past 30 years to treat and prevent breast cancer.

Several new antioestrogens have been developed over the recent past, which includes Toremifene, droloxifene, raloxifene, and idoxifene. Toremifene is a chlorinated analogue of tamoxifen and has similar site-specific oestrogen and antioestrogen activity. It has shown similar efficacy to tamoxifen with a response rate of 21% and a median survival of 38 months [Hayes DF]. However, substantial cross-resistance has been demonstrated with toremifene and tamoxifen [O'Regan RM]. Droloxifene, raloxifene and idoxifene are antioestrogens that are in clinical trials and all have binding profiles different from tamoxifen. Idoxifene has a binding capacity twice that of tamoxifen for oestrogen receptors and has been studied in phase I trials with partial response of about 14% with minimal toxicity [Coombes RC, 1995]. In animal models idoxifene use, decreased uterine weight, prevented bone loss, and lowered serum cholesterol levels [Nuttall ME]. Its role in the treatment of metastatic breast cancer needs further investigation. Raloxifene is a nonsteroidal benzothiophene derivative that binds to the

ER and has been classified as a SERM because it has been shown to have antioestrogenic effects in the uterus and breast as well as oestrogenic effects on bone and cholesterol [Black LJ]. Raloxifene though originally developed for breast cancer therapy was disappointing, but showed significant activity in the treatment and prevention of osteoporosis [Delmas PD]. However, the Multiple Outcomes of Raloxifene Evaluation (MORE) trial shows a reduced incidence of breast cancer in raloxifene-treated patients [Cummings SR]. Raloxifene reduces risk of invasive ER-positive breast cancer regardless of a woman's baseline breast cancer risk but does not reduce risk of noninvasive or ER-negative breast cancers. These results confirm those of the Multiple Outcomes of Raloxifene Evaluation, a previous randomised trial among women with osteoporosis [Grady D].

Fulvestrant (Faslodex) is an oestrogen receptor antagonist used in locally advanced or metastatic breast cancer following progression or recurrence on anti-oestrogen therapy. The Evaluation of Faslodex and Exemestane Clinical Trial (EFFECT) evaluated the efficacy and tolerability of fulvestrant and exemestane, in patients with locally advanced or metastatic breast cancer. Similar efficacy was seen in both treatment groups and there were no significant differences in reported adverse events between fulvestrant and exemestane [Gradishar W]. Three Phase III trials found no significant difference between fulvestrant and control, either anastrozole or exemestane, across efficacy and safety endpoints following prior endocrine therapy failure. Fulvestrant can therefore be considered as alternative therapy to AI in postmenopausal women with locally advanced or metastatic breast cancer that has recurred or progressed on prior adjuvant endocrine therapy [Flemming J].

## Ovarian ablation

Castration has been observed as an effective therapy for metastatic breast cancer for more than 100 years. Several different methods are available: surgical oophorectomy, radiation ablation and LHRH agonists [Block GE]. Oophorectomy is an irreversible surgical procedure, associated with morbidity and psychological trauma [Boyd S]. Ovarian irradiation is associated with delayed onset of action (up to 6 weeks) and a potential for damage to adjacent organs and tissues. Ovarian ablation reduced the annual odds of recurrence and death in breast cancer by approximately 25% [EBCTCG, 1996]. Studies have suggested temporary amenorrhea can confer benefit in early breast cancer, giving luteinizing hormone-releasing hormone (LH-RH) agonists an advantage over oophorectomy or radiation [Lonning PE, 2007]. Increasing interest has emerged in the role of ovarian function suppression, which has shown equivalence to adjuvant chemotherapy whether achieved by surgery or irradiation, [Tan SH]. The ABC Ovarian Ablation or Suppression Trial randomly assigned pre and perimenopausal patients with early-stage breast cancers, who were receiving prolonged (5 years) tamoxifen treatment with or without chemotherapy to ovarian ablation or suppression (by oophorectomy, ovarian irradiation, or treatment with luteinizing hormone-releasing hormone agonist) versus no ovarian ablation or suppression [ABC trial group, 2007]. Overall, no added effect of ovarian ablation or suppression was seen on relapse-free survival or overall survival of premenopausal women who were treated for early-stage breast cancer

## **Luteinizing hormone releasing hormone (LHRH) agonists**

LHRH analogues are an alternative to oophorectomy. These agents induce chemical castration and are effective in premenopausal patients with metastatic breast cancer [Filicori M]. Goserelin has been approved for therapy while buserelin and tryptorelin are under investigation. The continuous administration of LHRH agonists overstimulates the LHRH receptors in the anterior pituitary, causing an initial rise in follicle stimulating hormone (FSH) and luteinizing hormone (LH), followed by desensitisation of the receptors (1 to 2 weeks), leading to decrease in the release of the hormones [Dlugi AM]. Thus the decrease in gonadotrophins levels results in reduced drive to the ovaries, reducing the synthesis of oestrogen to postmenopausal level, within 4 weeks of starting an LHRH agonist and with discontinuation of treatment, serum oestradiol levels return to normal within 12 weeks [West CP]. Goserelin is administered as a monthly subcutaneous injectable implant that provides continuous release of the drug. Objective response rates have ranged from 31 - 63% in premenopausal women with advanced breast cancer. However, no difference in disease-free survival or overall survival rates has been shown in premenopausal patients treated with either goserelin or oophorectomy [Jonat W]. Toxicities associated with goserelin acetate are those consistent with a menopausal state, including hot flush, loss of libido, vaginal dryness and decreased bone mineral density. Trials are investigating the use of LHRH agonist in combination with an aromatase inhibitor (TEXT, SOFT and ABCSG 12 trial) in premenopausal patients [Huober J]. .

## Progestogens

Previously, used treatments such as progestogens (megestrol acetate and medroxyprogesterone acetate (MPA) are now rarely used or confined to fourth or fifth line treatments [Howell A, 2001]. Intermittent tamoxifen or intermittent/ alternated tamoxifen and MPA had no impact on overall survival as compared to classical continuous tamoxifen in patients with advanced breast cancer [EORTC study, 2006]. Two randomised, multicenter trials were conducted, involving a total of 764 patients comparing the efficacy and tolerability of anastrozole, and megestrol acetate in the treatment of postmenopausal women with advanced breast carcinoma whose disease had progressed after treatment with tamoxifen. Results demonstrated treatment with anastrozole 1 mg once daily results in a statistically and clinically significant advantage over a standard treatment, megestrol acetate [Eiermann W, 1998].

## **AROMATASE**

Aromatase also called cytochrome P-450 arom or oestrogen synthetase is a unique member of the cytochrome P450 superfamily. The enzyme is complex and consists of cytochrome P-450 haemoprotein and an NADPH reductase [Thompson EA].

### **Distribution**

Aromatase is mainly located in the endoplasmic reticulum of oestrogen producing cells. The enzyme is expressed in variety of cells and tissues including ovarian granulosa, testicular sertoli and leydig cells, placenta, adipose tissue, liver, muscle, skin fibroblasts, gonads and brain [Siiteri PK; Grodin JM]. A number of studies have reported aromatase activity both in normal breast tissue and in tumours [James VHT, Miller WR, 1987, Lipton A, 1987].

### **Mechanism of aromatisation**

Aromatase mediates the conversion of androgen into oestrogen, which is a last step in oestrogen biosynthesis. Aromatase catalyses the steroid hydroxylations involved in the conversion of androstenedione to oestrone or testosterone to oestradiol [Fishman J]. The two major androgens, androstenedione and testosterone serve as substrates for aromatase. The conversion of androgens to oestrogen involves three hydroxylation steps. The first two give rise to 19 hydroxy and 19 aldehyde structures and the third probably involves the C19 methyl group with release of formic acid. This enzymatic reaction results in desaturation of the A ring of the steroid molecule to produce an aromatic

structure and hence the enzyme is known as aromatase and reaction called aromatisation. Despite intense investigations, the precise mechanism involved in the final step has not been established definitively. The NADPH reductase is responsible for transfer of reducing equivalents from NADPH to cytochrome P450. The whole reaction requires 3mol molecular oxygen and 3mol NADPH per mol C19 steroid substrate metabolised. Two oxygen molecules are required for oxidation of C19 angular methyl group and the site of action of the third molecule is uncertain.

## **Aromatase gene**

Aromatase is expressed throughout the entire spectrum of the vertebrate phylum. The aromatase gene belongs to a separate gene family designated CYP19 as its overall homology to other members of the family is low [Harada N, 1990a]. The gene has been mapped to chromosome 15 and the structural organisation of CYP19 was studied by isolating and characterizing genomic DNA fragments containing the gene. The gene is larger than other members of the cytochrome P-450 super family and consists of 10 exons and spans at least 70Kb [Harada N, 1993]. It is now believed that that the gene exists in the human haploid genome as a single copy [Means GD]. The cDNA of the aromatase gene is 3.4 kb in size and encodes a polypeptide of 503 amino acids with a molecular weight of 55 kb.

## **Regulation**

There is only one gene for the aromatase but its expression is regulated in a tissue



specific manner, by the use of a number of different promoters [Simpson ER, 1993]. Aromatase transcripts are tissue specifically spliced by alternative usage of multiple exons I. Aromatase has been suggested to have multiple promoters attached to multiple exons I and to use tissue specific exons I and promoters, by alternative splicing mechanisms [Harada N, 1993]. The tissue specific exons I of the human aromatase gene are flanked with a unique promoter region containing the basic promoter sites and regulatory promoter or enhancer sites. Transcripts from placenta contain sequences that are at 35 kb upstream from the start of translation, under the regulation of distal promoter I.1. Transcripts from adipose tissue contain two different 5'termini – exon I.3 and exon I.4. Exon I.4 is situated just 100 bp upstream of exon II and exon I.4 20 kb downstream from exon I.1.

The tissue specific utilisation of alternative exons I can explain the complex regulatory mechanism of tissue specific expression of aromatase [Utsumi T]. In humans use of alternative and partially tissue-specific promoters in the placenta (promoter I.1), adipose tissue (promoters I.4, I.3, and II), skin (promoter I.4) and ovary (promoter II) regulates aromatase expression and is shown in **table–4**. Activation of these promoters and thus aromatase expression in these tissues is controlled by various factors [Simpson ER, 1993]. These include cytokines, cyclic AMP, glucocorticoids, phorbol esters and growth factors [Simpson ER, 1997]. In ovarian granulosa cells, follicle-stimulating hormone (FSH) stimulates the activation of promoter II via a cyclic adenosine monophosphate (cAMP)-dependent signaling pathway [Bulun SE, 1999a]. In adipose fibroblasts, glucocorticoids together with members of the interleukin-6 cytokine family give rise to activation of promoter I.4, whereas treatment with cAMP analogs or prostaglandin E<sub>2</sub>



(PGE<sub>2</sub>) switches the promoter use to I.3 and II in these cells [Chen S, 1999]. In the brain, a tissue-specific promoter 1F is used along with fibroblast (I.4)-type and ovarian (II)-type promoters [Sasano H, 1998b]. Cellular and molecular mechanisms responsible for the regulation of brain aromatase expression are not yet clear. However, regardless of the promoter used the encoded aromatase protein is identical in all these cells. Consequently, alternative promoter use, does not affect the structure of aromatase enzyme that catalyses the oestrogen formation. Thus an alternative splicing mechanism plays an important role not only in tissue specific expression but also in alteration of aromatase expression through developmental or neoplastic process of the same tissue [Utsumi T].

**Table 4: Tissue specific utilisation of alternative exons I of the human aromatase gene**

TISSUE	<u>Exon 1A</u>	<u>Exon 1B</u>	<u>Exon 1C</u>	<u>Exon 1D</u>	<u>Exon 1F</u>
	[1.1]	[1.2]	[1. 3]	[1.4]	[1. 5]
ADIPOSE TISSUE	-	++	-	-	-
PLACENTA	+++++	+	+	+	-
OVARY	-	-	++++	++++	-
PROSTATE	-	-	-	+	-
TESTIS	-	-	-	±	-
FOETAL LIVER	+	++++	+	+	±
ADULT LIVER	-	-	-	-	±
FOETAL BRAIN	-	±	-	-	+++
ADULT BRAIN	-	-	-	-	+

## **Adipose tissue expression and regulation**

MacDonald et al demonstrated that human adipose tissue was a major source of oestrogen production [1978]. Oestrogen production by adipose tissue increases as a function of obesity and aging. In both women and men with advancing age, there is a progressive increase in the efficiency of conversion of circulating androstenedione to oestrone [Hemsell DL]. In post reproductive years the degree of oestrogenisation is mainly dependent on the extent of adiposity, as the adipose tissue is the main source of oestrogen. The production of oestrogen correlated positively in both pre and postmenopausal women with excess body weight and increased as much as tenfold in morbidly obese postmenopausal women [MacDonald PC, 1978]. The messenger RNA, levels of aromatase and the activity per cell increased with advancing age [Bulun SE, 1999b; Cleland WH]. Both cell types found in adipose tissue, namely adipocytes and adipose stromal cells, express aromatase although much higher levels of expression has been observed with the latter [Sasano H, 1994a; Santen RJ, 1994]. Ackerman et al demonstrated aromatase expression in adipose stromal fibroblasts. Aromatase activity is higher in adipose tissue in patients with breast cancer than in women without the disease [Utsumi T] and highest in regions proximal to the tumour [Miller WR, 1991].

A number of agents alter aromatase expression in human adipose stromal cells in culture including glucocorticoids, cyclic AMP, phorbol esters and growth factors [Mendelson CR 1987]. The stimulatory effects of dexamethasone, requires the presence of foetal calf serum in the medium while dibutyl cAMP manifests an increase in activity only in the absence of foetal calf serum. Aromatase regulation by these agents is tissue specific

since glucocorticoids have no effect and phorbol esters inhibit cAMP induced aromatase activity in ovarian granulosa cells. Mahendroo et al have identified four P450 arom with unique 5'termini in adipose tissue and stromal cells in culture by RACE [rapid amplification of cDNA ends]. 1.3 specific sequence was expressed in cells in all conditions as well as in adipose tissue, 1.4 specific sequence was present only in tissue/cells stimulated with glucocorticoids and p II sequence was present only in cells stimulated with cAMP analogues as shown in **table 5**. Thus glucocorticoid stimulation of expression is mediated by promoter I.4, which would lead to the formation of transcripts containing exon I.4 in their termini and in contrast expression of promoter II specific sequences would be cAMP mediated [Mahendroo M]. It is uncertain whether additional factors are also required to produce the determined splicing pattern. Thus the 5'upstream region of this promoter contains a glucocorticoid response element and a gamma activating sequence (GAS) element, which can bind transcription factors for the signal transducer and activator of the transcription (STAT) family [Zhao Y]. Cytokines in the presence of glucocorticoids regulate aromatase gene expression via PI.4. Chen et al have proposed that in normal breast adipose tissue and fibroblasts, aromatase expression is driven by promoter 1.4 (glucocorticoid-dependent) and the action of promoters 1.3 and II is suppressed by the silence negative regulatory element. In cancer cells and surrounding tissue, the cyclic AMP level increases and aromatase promoters are switched to cyclic AMP dependent promoters 1.3 and II. Thus, regulation of aromatase in breast adipose tissue becomes abnormal during carcinogenesis due to switching of exons I and promoters, resulting in overproduction of oestrogen under

control of a new promoter. The role of alternative splicing pathway and the normal regulatory splicing can function as an on / off switch in gene expression.

**Table 5: Summary of major 5'termini in adipose cells and tissues**

<b>TISSUE CELLS</b>	<b>MAJOR 5' TERMINUS</b>
Adipose tissue	I.4, I.3
Adipose stromal cells in culture	I.3
Control – serum	I.3
Control + serum	I.3
Dex + serum	I.4, I.3
cAMP – serum	PII / I.3
cAMP + serum	PII / I.3
Ovary	PII
Placenta	I.1

## **Aromatase in oestrogen biosynthesis**

The conversion of androgens to oestrogen by aromatase is the last step in one of the three major steroidogenic pathways. In the premenopausal state, the major source of aromatase and of its substrates is the ovary. Extraglandular aromatisation of adrenal substrates also provide substantially to the oestrogen pool in the early follicular and luteal phases of the menstrual cycle [McNatty KP]. In the postmenopausal state, the ovary loses its complement of aromatase, although it does continue to secrete androstenedione [Naftolin F]. The adrenal gland provides the substrate for aromatase by directly secreting testosterone, androstenedione and dehydroepiandrosterone and its sulfate [Baird DT]. The peripheral tissues form the principal source of oestrogen after menopause [Grodin JM]. Unlike in the ovary, oestrogen production in adipose tissue is continuous and not cyclic. Synthesis of ovarian aromatase is regulated by an ovarian-pituitary (oestrogen-follicle-stimulating hormone) feedback loop, although a similar mechanism of aromatase expression in peripheral tissue has not been reported [Chen S, 1988].

## **Aromatase in breast cancer**

The association between increased aromatase expression and human breast cancer has been demonstrated previously [Miller WR, 1991; Macaulay VM; Sasano H, 1998c]. Approximately two-thirds of human breast cancers contain appreciable levels of aromatase activity [Silva MC] and synthesise biologically significant amounts of oestrogen locally within the tumour [Miller WR, 1987]. Reported aromatase activity

ranges between 5 and 80 pmols/g protein/hour equivalent to 0.02 - 0.9% conversion of androstenedione to oestrone [Dowsett M, 1996]. Demonstration that aromatase activity contributes significantly to intratumoural oestrogen concentrations of breast carcinoma has been directly obtained by injection of  $^3\text{H}$  androstenedione and  $^{14}\text{C}$  oestrone prior to surgical sampling of the tumour [Reed MJ]. There is evidence that aromatase expression is increased in the breast quadrant in which the tumour is located [O'Neill; Purohit A]. Therefore breast tumours may arise in quadrants with high aromatase activity or they may secrete factors which stimulate aromatase activity in tissues proximal to the tumour. The cell type and their relative contribution to aromatase activity in human breast tissue remains a controversial issue with evidence of aromatase either in epithelial cells, stromal cells or both cell types. Santen et al observed, the highest degree of aromatase expression occurred in stromal spindle cells, while tumour epithelial, stromal, inflammatory and normal breast elements contained lesser amounts [1994]. Furthermore, a statistically significant correlation was found between biochemical measurements of aromatase and the stromal spindle cell histological score [Santen RJ, 1998]. Immunocytochemical studies revealed that aromatase is mainly expressed in the stromal adipocytes [Lu Q]. Heterogeneity in aromatase expression by human breast cancers has also been reported by other investigators [Harada N, 1995, Lu Q, Zhang Z]. Increase aromatase protein expression in tumours was observed by Lu et al, while Bulun et al found highest mRNA transcript levels of aromatase in the tumour bearing quadrant [1994b]. In aggregate, observations provide further support for the possibility that human breast tumours may contain biologically relevant amounts of aromatase, producing oestrogen exerting autocrine or paracrine effects [Eppenberger US]. The



interest in intratumoural aromatase activity is that it could form the most important source of oestrogen for the tumour and thus the most important target for inhibition.

## **AROMATASE INHIBITORS**

Recently, several new aromatase inhibitors have been developed with greater clinical efficacy and improved tolerability [Miller 2005]. These agents including exemestane, letrozole and anastrozole are highly selective and potent inhibitors of aromatase. Promising results on the third-generation aromatase inhibitors (AIs), anastrozole, letrozole and exemestane in advanced disease, led to the development of these agents in the treatment of early breast cancer in both adjuvant and neoadjuvant setting.

### **Classification**

Aromatase inhibitors are classified into steroidal (type I) and non-steroidal inhibitors (type II). They differ in their mode of interaction with the aromatase enzyme. Aromatase inhibitors can also be classified based on their generation (**table- 6**). The third generation nonsteroidal aromatase inhibitors are triazoles. The new inhibitors have greater specificity and potency than earlier generations.

**Table – 6: Classification of aromatase inhibitors according to the generation**

<b>GENERATION</b>	<b>NON STEROIDAL INHIBITORS</b>	<b>STEROIDAL INHIBITORS</b>
<b>FIRST</b>	AMINOGLUTETHIMIDE	
<b>SECOND</b>	FADROZOLE	FORMESTANE
<b>THIRD</b>	ANASTROZOLE LETROZOLE	EXEMESTANE

## Mechanism of action

Type I inhibitors interact with the substrate binding site of the enzyme and invariably have an androgen structure while type II inhibitors interfere with the cytochrome P450 moiety of the system [Miller WR, 1989].

## Steroidal inhibitors

Steroidal inhibitors include: **exemestane and formestane**. Steroidal inhibitors compete with the endogenous substrates, androstenedione and testosterone for the active site of the enzyme, where they act as a false substrate and are processed to intermediates that bind irreversibly to the active site, causing irreversible enzyme inhibition. This type of inhibition is mechanism based inhibition or suicide inhibition. Because the inhibitor needs to be activated by the enzyme, the inhibition is time dependent, but the effect is generally long term and specific.

## Nonsteroidal inhibitors

Nonsteroidal (type II) compounds are structurally imidazoles and triazoles and include: **aminoglutethimide, fadrozole, letrozole and anastrozole**. Nonsteroidal inhibitors interact with the cytochrome P450 moiety of the aromatase enzyme complex at the same time excluding the endogenous substrate from the active site, where they form a coordinate bond to the haem iron atom. The drugs bind reversibly and inhibition is maintained only in the continuous presence of the drug. Imidazoles have a shorter half life than the triazoles, potentially resulting in lower invivo efficacy, despite similar

invitro potency [Brodie AMH, 1994]. Many type II drugs have the disadvantage of poor specificity, in that a variety of enzymes possess cytochrome P450 prosthetic groups and may be inhibited [Santen RJ, 1981]. However, the amino acid sequence of P450 arom shows it to be quite distinct from other members of the cytochrome family and more specific aromatase inhibitors are available now.

### **Aminoglutethimide**

Aminoglutethimide was first used as an anticonvulsant in 1967 [Cash AJ]. It was later used to for medical adrenalectomy in breast cancer patients [Santen RJ, 1981]. This inhibitor lacks selectivity for the aromatase enzyme and has effects on other cytochrome P450 enzymes. Aminoglutethimide therapy is associated with many drug interactions and also causes adrenal insufficiency, requiring the concurrent administration of glucocorticoids [Johanessen DC]. This along with other adverse effects including somnolence, rash and nausea served to limit the usefulness of aminoglutethimide [Goldhirsch A].

### **Anastrozole**

Anastrozole (Arimidex: ICI-D1033; 2, 2'-5-1H-2, 4-triazol-1-ylmethyl-1, 3-phenylene bis 2-methyl-propioononitrile) is a potent and selective benzyl triazole derivative [Buzdar AU, 1996b]. It is well absorbed from the gastrointestinal tract, excreted through hepatic metabolism (85%) with a half-life of 50 hours. It has no effect on cortisol or aldosterone secretion and requires no steroid replacement therapy [Plourde PV, 1995].

Geisler et al examined the effect of anastrozole on *invivo* aromatisation and plasma oestrogen levels. Both 1 and 10mg doses inhibited *invivo* aromatisation by 96.7% and 98.1% respectively. The drug also significantly suppressed plasma oestradiol (84%), oestrone (87%), and oestrone sulfate (94%), irrespective of the dose studied. Kleeberg compared the effects on oestrogen suppression between anastrozole and formestane in postmenopausal women with advanced breast cancer and observed that it was more consistent with anastrozole. Two large randomised phase III trials have compared anastrozole to megestrol acetate in the treatment of advanced breast cancer in postmenopausal women who had failed tamoxifen therapy [Buzdar AU, 1996a]. The combined analysis demonstrated a statistically significant survival advantage with 1mg dose of anastrozole compared with megestrol acetate (HR 0.78,  $p < 0.025$ ). The 10mg daily dose of anastrozole also had a survival advantage, but not statistically significant. The incidence of adverse drug effects in these trials was low, but weight gain and dyspnoea were significantly greater in the megestrol acetate group compared to the anastrozole group ( $p = 0.045$  for weight gain,  $p = 0.022$  for oedema). The 1mg dose was selected for FDA approval and for future trials, as the 10mg dose had more adverse effects with no increased clinical efficacy.

### **Adjuvant setting**

Anastrozole was compared with tamoxifen for 5 years in 9366 postmenopausal women with localised breast cancer in the Arimidex, Tamoxifen Alone or in Combination (ATAC) study [ATAC trial]. After a median follow-up of 68 months, anastrozole significantly prolonged disease-free survival (575 events with anastrozole vs 651 with tamoxifen, HR 0.87,  $p = 0.01$ ), time to recurrence (402 vs 498, HR 0.79,  $p = 0.0005$ ),

significantly reduced distant metastases (324 vs 375, HR 0.86,  $p=0.04$ ) and contralateral breast cancers (35 vs 59, 42% reduction,  $p=0.01$ ). Anastrozole was associated with fewer side-effects than tamoxifen, especially gynaecological problems and vascular events; but were associated with increase in arthralgia and fractures. The ATAC trial demonstrated that adjuvant endocrine therapy for postmenopausal patients with early stage breast cancer, anastrozole was superior to tamoxifen in terms of disease-free survival (DFS), time to recurrence (TTR), and incidence of contralateral breast cancer (CLBC). A recent analysis of annual recurrence rates at 2.5 and 5 years found that the majority of excess first events in patients receiving tamoxifen occurred during the first 2.5 years of therapy, and although the greatest difference between anastrozole and tamoxifen appeared early at about 2 years, the data indicate that most of the early difference is not in reduction of distant metastatic events and the difference in distant disease is greater at 5 years than at 2.5 years (16% vs. 7%) [Houghton J]

### **Sequential therapy**

The ABCSG8 trial randomised patients to either 5 years of tamoxifen or 2 years of tamoxifen followed by 3 years of treatment with anastrozole. Although there was a 24% relative reduction in recurrence risk with anastrozole, the difference between anastrozole and tamoxifen treatment arms for the primary end point, event free survival, was not statistically significant ( $p = 0.07$ ) at 30 months follow-up [Jakesz R].

### **Letrozole**

Letrozole (4, 4'-1H-1, 2, 4-triazol-a-yl-methylene-bis-benzonitrile) is a potent oral, reversible, nonsteroidal competitive aromatase inhibitor. It is rapidly and completely

absorbed from the gastrointestinal tract, metabolised to an inactive metabolite and excreted renally. The elimination half-life of letrozole is about 2 days and steady-state plasma concentration is reached in 2-6 weeks. It is a potent inhibitor of peripheral aromatase activity (> 98%) and suppresses plasma oestrogen levels by greater than 95% [Klein KO].

Bajetta et al evaluated the effects of two doses of letrozole (0.5 mg and 2.5 mg orally daily) on oestrogen suppression in a double blind, randomised trial involving postmenopausal patients with advanced breast cancer progressing after tamoxifen. A significant suppression of oestrone and oestradiol levels was achieved by both letrozole doses without affecting adrenal activity. Two large randomised trials have evaluated the role of letrozole in the second-line treatment of advanced breast cancer. Gershanovich et al, compared letrozole and aminoglutethimide in 555 postmenopausal women with advanced breast cancer, previously treated with antioestrogens. Letrozole 2.5 mg was superior to aminoglutethimide in time to progression, time to treatment failure and overall survival. Treatment-related adverse events occurred in 33% of letrozole patients compared with 46% of aminoglutethimide patients. In another large phase III trial, letrozole was compared to megestrol acetate as second-line therapy in postmenopausal women with advanced breast cancer previously treated with antioestrogens [Dombernowsky P]. The differences in overall recurrence rate were statistically significant between the letrozole 2.5 mg group compared with both the megestrol acetate group ( $p=0.04$ ) and the letrozole 0.5 mg group ( $p=0.004$ ). The duration of response was significantly longer for letrozole 2.5 mg compared with the megestrol acetate group ( $p=0.02$ ).



Letrozole 2.5 mg was also significantly superior to megestrol acetate and letrozole 0.5mg in time to treatment failure. It was significantly better tolerated than megestrol.

### **Neoadjuvant setting**

In a double blind trial, 337 postmenopausal women with hormone receptor positive locally advanced breast cancer were randomised to either letrozole (2.5mg) or tamoxifen (20mg daily) for 4 months [Eiermann W]. Tumour response rates were higher with letrozole (55% vs 36%,  $p < 0.001$ ) and more patients on letrozole had sufficient tumour response to allow breast conserving surgery (45% vs 35%,  $p = 0.022$ ).

### **Adjuvant therapy**

The Breast International Group (BIG) 1-98 phase 3, double-blind, controlled trial enrolled 8010 postmenopausal women with early breast cancer. The BIG 1-98 trial had a unique trial design, as it randomised patients to: 5 years adjuvant tamoxifen, or 5 years of adjuvant letrozole, or to one of the two sequential arms where therapy was initiated with either tamoxifen or letrozole and subsequently switched to the alternative treatment after 2-3 years so that patients received a total of 5 years of endocrine treatment. The primary core analysis included events in the two monotherapy arms as well as events up to the time when patients were to switch in the two sequential arms. After a median follow-up of 25.8 months, letrozole significantly increased DFS by 19% when compared with tamoxifen (HR 0.81;  $p = 0.003$ ), with an absolute benefit of 2.6% at 5 years (84% letrozole vs. 81.4% tamoxifen). Initial adjuvant letrozole was particularly effective in reducing the relative risk of distant recurrence by 27% (HR 0.73;  $p = 0.001$ ). Overall, in the BIG 1-98 trial, there were fewer deaths in the letrozole group than in the tamoxifen group (166 or 4.1% vs. 192 or 4.8%, respectively), but survival advantage was not seen.

After a median follow-up of 2 years, letrozole markedly reduced the risk of distant metastatic events (87 vs. 125 events, 30% reduction) [Thurlimann B, 2005].

### **Extended therapy**

A double-blind, placebo-controlled trial was conducted to test the effectiveness of five years of letrozole therapy in postmenopausal women with breast cancer who have completed five years of tamoxifen therapy [Goss PE]. The primary end point was disease-free survival. A total of 5187 women were enrolled (median follow-up 2.4 years). At the first interim analysis, there were 207 local or metastatic recurrences of breast cancer or new primary cancers in the contralateral breast; 75 in the letrozole group and 132 in the placebo group with estimated four-year disease-free survival rates of 93 % and 87 %, respectively, in the two groups ( $p < 0.001$ ). A total of 42 women in the placebo group and 31 women in the letrozole group died ( $p = 0.25$  for the comparison of overall survival). Letrozole therapy after the completion of standard tamoxifen treatment significantly improves disease free survival. Low-grade hot flush, arthritis, arthralgia, and myalgia were more frequent in the letrozole group, but vaginal bleeding was less frequent. Osteoporosis was seen in 5.8 % of the women in the letrozole group and 4.5 % of the women in the placebo group ( $p = 0.07$ ) but the fracture rates in the two groups were similar.

### **Fadrozole**

Fadrozole (CGS 16949A: 4-5, 6, 77, 8-tetrahydroimidazole 1,5a-pyridin-5yl benzonitrile monohydrochloride) is an inhibitor of aromatase with an inhibitory constant of  $K_i$  of

0.19nm. Fadrozole at a dose of 1 mg twice daily causes maximal suppression of plasma and urinary oestrogens [Dowsett M, 1990]. It may also cause a degree of inhibition of cortisol and aldosterone production, although the clinical relevance of this is unclear [Dowsett M, 1994]. A phase II study compared fadrozole with megestrol acetate and approximately 20% of postmenopausal women with metastatic cancer responded to Fadrozole [Buzdar AU, 1996a]. No significant difference was observed in response rate and time to progression, response duration or survival. It has also been compared to tamoxifen as first line of therapy for metastatic cancer, with statistically no significant difference in response rate and duration [Falkson CI]. Fadrozole has been approved for treatment in Japan.

## **Formestane**

Formestane (Lentaron; 4-OHA; 4-Hydroxyandrost-4-ene-3, 17- dione) is a structural analogue of androstenedione and a highly specific aromatase inhibitor [Lonning PE, 1998].

Formestane is a selective inhibitor of the aromatase enzyme and is available only in injectable form [Coombes RC, 1992]. It has low toxicity and adverse effect includes hot flushes, GI disturbances and alopecia [Coombes RC, 1987]. Parenteral administration produces 65% oestradiol suppression and 85% peripheral aromatase inhibition [Dowsett M, 1994]. Studies have revealed that formestane has no oestrogenic or antioestrogenic properties but has androgenic effects under certain circumstances [Brodie AMH, 1986]. Data from phase II clinical trials have demonstrated 33% objective regression rate in

postmenopausal women previously treated with multiple endocrine therapies [Bajetta E, 1997].

A phase III randomised trial of formestane versus tamoxifen was carried out in 409 postmenopausal women with advanced breast cancer [Perez CR, 1994]. 33% had objective response to formestane compared to 37% to tamoxifen ( $p=0.48$ ) with median response of 15 vs 20 months ( $p=0.17$ ) and median survival of 35 vs 38 months ( $p=0.64$ ). However time to disease progression was longer for tamoxifen (213 vs 294 days) but both drugs were well tolerated. Formestane is now approved in Europe for the treatment of metastatic breast cancer in women who have failed tamoxifen therapy.

## **Exemestane**

Exemestane (6methylene-androsta-1, 4-diene-3, 17 dione) is an irreversible aromatase inhibitor [Evans TR] developed for oral use. Its  $K_i$  is 10.2 nmol/l for competitive inhibition and 26 nmol/l for irreversible inactivation. Exemestane is selective and does not interfere with cortisol or aldosterone concentration. In general, exemestane is well tolerated [Lonning PE, 1997].

In phase II trial involving 134 patients with breast cancer resistant to tamoxifen, the overall response rate was 22% [Kvinnsland S]. Median duration of objective response was 68 weeks and time to progression was 29 weeks. In a phase III randomised, multicentre, double-blinded study, exemestane was compared with megestrol acetate (MA) in 769 postmenopausal women with advanced breast cancer refractory to tamoxifen therapy [Kaufmann M, 2000]. There was no significant difference between the two groups in neither baseline characteristics nor prognostic factors.

Exemestane was significantly better than MA in terms of median time to progression (4.7 vs. 3.8 months), duration of overall success (13.8 vs. 11.3 months) and overall survival. Furthermore, the study showed that exemestane was better tolerated than MA, with a withdrawal rate of 1.4% (vs. 2.5% with MA). Reported common side effects includes, nausea (9.2%), fatigue (7.5%) and hot flushes (6%). The number of patients gaining weight was significantly lower in the exemestane arm (7.6 vs. 5.1%,  $p = 0.001$ ).

### **Switch therapy**

The exemestane intergroup study conducted a randomised double blind, trial was conducted to test whether switching to exemestane, after two to three years of tamoxifen therapy, was more effective instead of continuing tamoxifen for five years. The primary end point was disease-free survival. Of the 4742 patients enrolled, 2362 were randomly assigned to switch to exemestane, and 2380 to continue to receive tamoxifen. After a median follow-up of 30.6 months, 449 first events (local or metastatic recurrence, contralateral breast cancer, or death) were reported: 183 in the exemestane group and 266 in the tamoxifen group. The unadjusted hazard ratio in the exemestane group compared to the tamoxifen group was 0.68 ( $p < 0.001$ ), representing a 32 % reduction in risk and corresponded to an absolute benefit in terms of disease-free survival of 4.7 % at three years after randomisation. Overall survival was not significantly different in the two groups, with 93 deaths occurring in the exemestane group and 106 in the tamoxifen group. Contralateral breast cancer occurred in 20 patients in the tamoxifen group and 9 in the exemestane group ( $p = 0.04$ ). Severe toxic effects of exemestane were rare. Exemestane after two to three years of tamoxifen significantly improved disease-free survival compared to the standard treatment for five years with tamoxifen.

## Conclusion

Third-generation AIs are now an integral part of hormonal therapy for postmenopausal patients with endocrine-sensitive breast cancer. Gradually, tamoxifen has been replaced by the third-generation aromatase inhibitors (AIs)—anastrozole, letrozole, and exemestane. All studies consistently show the superiority of these agents over tamoxifen. For adjuvant therapy, the recent American Society of Clinical Oncology guidelines advise that AI treatment should be included in the overall therapy of early breast cancer but there is no one specific AI, which has been, recommend over the other [Winer EP]. However, the best therapeutic strategy for AIs in an adjuvant setting remain to be defined, in particular in terms of the role of the sequential approach and the duration of therapy beyond 5 years. The overall therapeutic index of AIs appears to be superior to that of tamoxifen with proven improved efficacy and a better toxicity profile. In terms of safety, regardless of the adjuvant treatment setting, bone loss, increased fracture rates, and other musculoskeletal disorders are the most serious side effects associated with the use of AIs [BIG, ATAC and IES study]. However, studies have shown that these bone health issues can be easily managed and the impact of AIs on bone health minimised with concomitant zoledronic acid use if necessary [Bundred N]. By comparison, toxicities commonly associated with tamoxifen, such as thromboembolic events and endometrial abnormalities, are more serious and less easily managed.

It is reasonable to offer adjuvant AI therapy at the earliest opportunity due to the higher rate of recurrences, adverse events, and withdrawals associated with adjuvant tamoxifen over the first 2 years of treatment [Houghton JJ]. For patients currently receiving

adjuvant tamoxifen therapy, it is clear that they will be better protected against a relapse if they are switched to an AI. Finally, for patients currently completing adjuvant therapy with tamoxifen, initiating extended adjuvant letrozole offers the only proven opportunity to further protect against late recurrence.



## **MATERIALS AND METHODS**

### **Materials**

Placenta, fibroblasts cell culture, malignant and nonmalignant breast tissues were used to measure aromatase activity and to study the effects of aromatase inhibitors. All patients had given their informed consent to participate in the study (approved by the Hospital Trust Ethics Committee)

### **Placenta**

Placenta was obtained from women immediately following delivery. It was washed, chopped into small pieces and stored in aliquots of 500mg in liquid nitrogen. The placenta was used as control system and the experiments were conducted in duplicates.

### **Culture of fibroblast cell lines from human breast tissue**

Adipose tissue was obtained from patients undergoing mastectomy for the treatment of breast cancer. The gross fat obtained from mastectomy specimens were collected and transferred to the laboratory in an aseptic manner in sterile phosphate buffer saline in ice. The tissue was processed immediately after collection.

### **Collection of breast tissue**

Postmenopausal women with large (>3cm), oestrogen receptor rich (>20 fmol/mg cytosol protein) primary breast cancers (stages T2, T3, N0, N1) were studied. All patients received primary endocrine therapy comprising aromatase inhibitors with either



letrozole, anastrozole or exemestane. None had previously received treatment with hormonal agents for breast cancer or were taking hormone preparations at the time of the study. Tumour and adjacent nonmalignant tissues (when possible) were obtained by surgical biopsy before commencing treatment and at the time of definitive surgery (3 months). All tissues were put on ice in the operating theatre and transported to the laboratory. The tissues were stored in liquid nitrogen.

## **2.1.1 CELL CULTURE**

### **2.1.1 CELL LINES**

#### **2.1.1a Cell culture media**

The fibroblast cell lines were cultured in Minimal Essential Medium (alpha MEM) and supplemented with 15% v/v of heat inactivated foetal calf serum (FCS), Penicillin (100 µg/ml) and Streptomycin (100 µg/ml).

#### **2.1.1b Culture of fibroblast cell lines**

The cell lines were grown in monolayer culture in a humidified incubator maintaining an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. All tissue culture procedures were carried out in a laminar flow cabinet using aseptic techniques and using sterile, disposable plastic pipettes and tips. To ensure a sterile environment, the laminar flow cabinet was thoroughly cleaned with a solution of 70% ethanol before any operations were conducted. In addition all cell lines were handled using disposable latex gloves.

#### **2.1.2a Enzymatic dissociation of fibroblast cells**

The fat was washed twice in 15 mls of Dulbecco 'A' phosphate buffered saline (PBS) and chopped into small pieces using sterilised scissors and forceps. Aliquots of 2g were then placed into universal containers and minced. A sterile filtered collagenase solution (600 units/ml) in alpha MEM equivalent to 5ml per gram of tissue was mixed with the tissue and the universal container was placed in a shaking incubator at 37°C. The tissue

was incubated for 30 minutes with gentle agitation. The contents were allowed to settle for 10 minutes and the liquid phase was aspirated from below the surface lipid layer and centrifuged for 10 minutes at 3000 rpm. The supernatant was decanted and the resultant pellet resuspended by inversion in 20 mls of Dulbecco 'A' PBS to wash out the collagenase. The cells were again centrifuged at 3000 rpm for 10 minutes and washed in Dulbecco 'A' PBS. These steps of centrifugation and washing were repeated twice, prior to suspension of the pellet in alpha minimal essential medium supplemented with 15% heat inactivated foetal calf serum, Penicillin and Streptomycin.

### **2.1.2b Primary Cell Culture**

The cells were resuspended in 2ml of growth medium per gram of original tissue and plated out in a 6 well plate. Each well was used for 2 grams of tissue. The cultures were incubated at 37°C for 48 hours in an atmosphere of 5% CO<sub>2</sub> - 95% air at 37°C. After 48 hrs, cultures were washed twice with PBS and fresh media added. Media were changed every 2-3 days until the cells grew to confluence (2-3 weeks).

### **2.1.2c Secondary Cell Culture**

Continuous cell growth was maintained through regular subculture. After achieving 90% confluence cells were harvested through trypsinisation, diluted 1:4 in medium and seeded in 6 well dishes. The subculture process involved aspiration of spent medium from cells followed by 2 washes with Dulbecco A phosphate buffered saline (PBS) and a 3 minute incubation at 37°C in 2ml trypsin EDTA (0.2ml of 10 x trypsin in 1.8ml of

Dulbecco A PBS). The cells were then detached through gentle agitation and 10ml of appropriate media were added to the cell suspension. Following centrifugation at 2000 rpm for 5 minutes, resuspension of the pellet in 10ml of medium, repeated pipetting ensured a single suspension. 2ml of this suspension were delivered into each well. The cells were allowed to adhere over a 24-hour period before being supplemented with fresh medium. There after the medium was changed every 48 hours. Beyond passage one, the cells displayed a reduced growth rate and adherence to the plastic wells.

#### **2.1.2d Long term storage of cells**

The monolayers were washed twice in PBS and incubated with trypsin EDTA solution (1mls) with gentle agitation until the cells had dispersed. The cells were then pelleted by centrifugation at 2000 rpm for 10 minutes. The pellet was then resuspended in 1 ml of freezing medium, which consisted of 5% dimethyl sulphoxide (DMSO) in FCS. The cells were then mixed with the freezing medium by titration, before being transferred to cryogenic ampoules and placed in the  $-70^{\circ}\text{C}$  freezer overnight. The cells were then transferred to the gas phase, liquid nitrogen store.

#### **2.1.2e Recovery of cells**

Frozen cells were recovered by thawing the vial at  $37^{\circ}\text{C}$  in the water bath and media were added drop wise with gentle mixing for 10 seconds until twice the original volume was added. The suspensions were transferred to 15 ml conical tubes, 10 ml of media added with gentle mixing of the tube contents and centrifuged X 2 at 2000 rpm for 5

minutes. The resultant pellet was resuspended in growth medium and transferred into 6 well plates.

### **2.1.2f Preparation of cell pellets**

The cells were washed with phosphate buffer saline. Trypsin (1ml) was added to the culture plate and the cells were allowed to detach from the plastic. The cells were then transferred to a test tube and the culture plates were washed with PBS (X2). The tubes were centrifuged for 10 minutes at 3000rpm. The pellets were resuspended in 0.5mls of PBS and the cell count was carried using a haemocytometer.

### **2.1.2g Manual determination of cell numbers**

Cells to be counted were incubated with 1ml of trypsin EDTA for 2 minutes at 37°C and washed twice with Dulbecco 'A' phosphate buffered saline. The suspension was transferred to a 10ml test tube centrifuged for at 3000 rpm 10 minutes. The pellets were resuspended in 0.5ml of PBS and the cell numbers determined using both chambers of a haemocytometer (five 1mm squares in each chamber).

### **2.1.2h Aromatase assay**

Aromatase activity was determined by 'tritium release assay' and the protocol used is an adaptation of that described by Frieden et al. It is based on the measurement  $^3\text{H}$  water after incubation with  $1\beta^3\text{H}$   $\Delta^4$ -androstenedione.

The fibroblast cell cultures were allowed to grow to confluence. The culture medium was removed and the cells washed twice in Dulbecco 'A' PBS. Two blanks were used as a control, which consist of dishes containing media but no cells. To each 6 well plate, 4 ml of reaction mixture was added and the plates were incubated for 5 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> - 95% air. The reaction was stopped by placing all dishes in ice for 15 minutes. Media were aspirated (4mls) and added to ice cold chloroform (5mls) in 10ml glass test tubes. Each test tube was shaken thoroughly (x100 times) and centrifuged at 2000 rpm for 5 minutes. Triplicate aliquots (10µl) of 1a-3H-androst-4-ene-3, 17-dione were added to counting vials to measure the total counts and calculate the specific activity of the precursor. Charcoal pellets were prepared by centrifuging 1ml of charcoal suspension (2000rpm for 15 minutes) in 12 mm x 75mm glass tubes and aspirating the supernatant. Duplicate aliquots (1.5ml) of aqueous phase were removed and added to tubes containing charcoal pellets and vortexed. The tubes were allowed to stand for 10 minutes with occasional mixing and then centrifuged at 3000 rpm for 15 minutes. Supernatant (1ml) from one duplicate was transferred into a counting vial (A) containing NE260 liquid scintillant (10ml). Supernatant (1 ml) from the other duplicate was transferred into a counting vial (B) and evaporated to dryness before the addition of 10 ml of NE260. All vials were vortexed and their radioactivity measured using Tri-Carb 11900 CA liquid scintillation analyser.

## **2.2 EXVIVO STUDIES - BREAST TISSUE**

### **2.2.a Preparation of particulate fraction**

All the procedures were performed in liquid nitrogen unless stated otherwise. Tissue was cut into small pieces and pulverised using a mikro dismembranator. The dry weight was determined. The tissue was homogenised by hand in an all glass uniform homogeniser with a 2ml of 0.1 M phosphate buffer. The homogenate was transferred to a 10ml conical glass tube, washing out the homogeniser with a further 10ml buffer, vortex mixed and centrifuged at 800g for 5 minutes (1900 rpm using a GPR centrifuge with GH-3.7 rotor). The supernatant was decanted into thick wall polycarbonate tube and centrifuged at 10,000g for 60 minutes (50,000 rpm using an L7 ultracentrifuge with 70 Ti rotor). The pellet (particulate fraction) was resuspended in 2.4 mls of buffer using a soniprep 150 ultrasonic disintegrator in short bursts.

### **2.2.b Purification of androst-4-ene-3,17-dione**

1  $\beta$ -<sup>3</sup>H androst-4-ene-3, 17-dione, (100  $\mu$ ci/ml 3.61nmol/ml) was purified by thin layer chromatography on silica gel using chloroform: acetone (185:15). It was dissolved in ethanol to a concentration of 100 $\mu$ Ci/ml. Duplicate aliquots (10 $\mu$ l) were transferred to the incubation mixture, into 60x27mm glass counting vials containing 10ml NE260 liquid scintillator for measurement of the specific activity of the precursor.

### **2.2.c Preparation of radioactive $^3\text{H}$ androst-4-ene-3,17-dione**

Androst-4-ene-3, 17-dione 22ng/ml in buffer

Prepared from a 1mg/ml ethanolic solution as follows:

1. Diluted 0.1 ml to 10 ml with buffer to give a 10 $\mu\text{g/ml}$  solution
2. Diluted 0.1 ml of solution (1) to 4.4 ml with buffer to give a 227ng/ml solution

### **2.2.d Aromatase assay for particulate fraction**

To 1.1ml of cofactor/precursor mixture, prewarmed in a 50x12 mm flat bottom glass tube, 0.5 ml of particulate fraction was added, vortex mixed and incubated at 37°C for 5 hours in a shaking water bath. A reagent blank was set up by substituting 0.5 ml of buffer for the particulate fraction. The incubation was stopped by decanting the mixture into

3 ml, of ice-cold chloroform in a 10 ml round bottom glass tube and shaking vigorously. After centrifugation at 2000g for 3 minutes (3000 rpm using a GPR centrifuge with GH 3.7 rotor), the chloroform layer was discarded using a pasteur pipette and the aqueous layer was re-extracted with a further 3 ml chloroform. Duplicate aliquots (0.8ml) of the aqueous phase were transferred into 75x10mm round bottom glass tubes containing 1ml of 5% charcoal suspension. Tubes were vortex mixed and incubated at 4°C for 10 minutes with occasional further mixing, then centrifuged at 2000g for 15 minutes (3000rpm).

Supernatant (0.5 ml) from each tube in triplicate was transferred into a 60x27mm low background glass counting vial (A) containing 10ml NE260 liquid scintillator. All vials



were vortex mixed and their radioactivity measured using a Tri-carb 1900 CA liquid scintillation analyser.

Radioactivity associated with  $^3\text{H}$  release into the aqueous phase was calculated as follows:

$$\text{Aqueous phase activity (dpm)} = [(A-B) \text{ sample} - (A-B) \text{ blank}] \times \frac{1.1}{0.8} \times \frac{0.8}{0.5} \text{ (dpm)}$$

where A = mean of  $A_1$ ,  $A_2$  and B = mean of  $B_1$ ,  $B_2$

## 2.2. e Protein assay

Protein concentration was determined using a Bio-Rad protein assay kit which followed the method of Bradford (1976). The protocol followed is detailed below:

1ml of protein assay dye reagent in triplicate 12x75mm tubes, was added to 20 $\mu\text{l}$  of buffer (blank), particulate fraction (sample) or BSA standards (0.1-0.8 mg/ml). Tubes were vortex mixed, incubated for 5 mins at 20°C and 200 $\mu\text{l}$  of the reaction mixture transferred to the wells of a micro titre plate. The absorbance of sample and standards was measured at 600nm against the blank using a model 222550 EIA plate reader. The protein concentration of the particulate fraction was determined from the standard curve for BSA using an assay calculation program [Biosoft, Cambridge] and Macintosh IICS [Apple computer Inc, Cupertino, California].

## **Verification of assay**

To determine the effects of aromatase inhibitors on aromatase activity in cultured fibroblasts, experiments were conducted in duplicate cultures using blanks (plates with media but no cells) as control, fibroblasts and with letrozole (100nm). Aromatase activity was measured by tritium release assay as described before. The duplicate samples were evaporated under a stream of nitrogen to eliminate any tritiated aqueous products and aromatase activity was measured in paired samples before and after evaporation.

## **Induction of aromatase activity in cell culture**

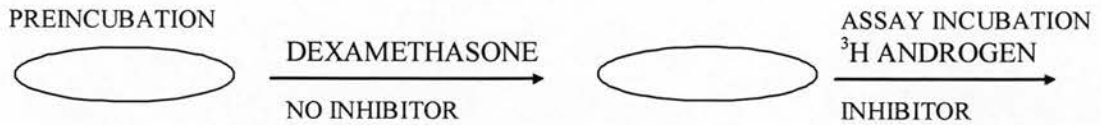
Aromatase activity was induced by incubating the cell lines with dexamethasone (1 $\mu$ M) in the presence of 15% FCS for 18 hours prior to the assay.

## **Study design I**

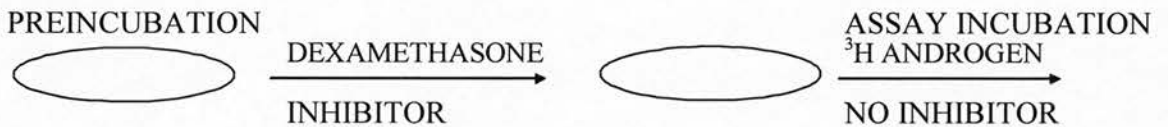
Two different study methods were explored:

**Cultures were preincubated in the absence of aromatase inhibitors [18 hrs] and assayed in its presence of various aromatase inhibitors – letrozole, anastrozole, aminoglutethimide, fadrozole, formestane and exemestane.**

- The sequence of preincubating cultures with dexamethasone (to induce aromatase) and then adding inhibitors during the assay procedure parallels in situ studies in patients whom assays are performed in the presence of the drug.



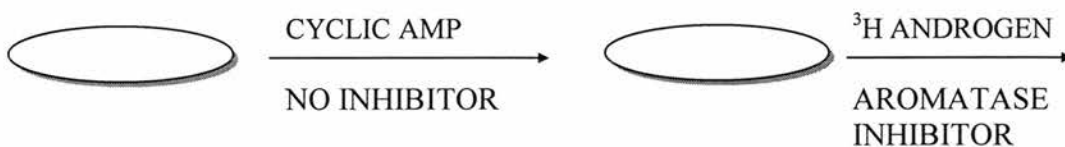
- Cultures were preincubated in the presence of aromatase inhibitors (18 hrs) and assayed in its absence. This parallels the ex vivo study where the tumour is removed from the presence of the drug.



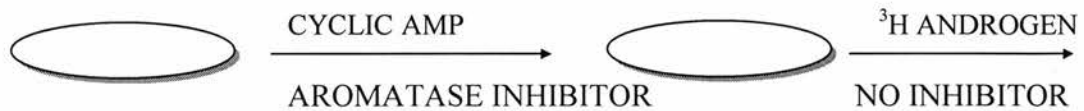
## Study design II

Cyclic AMP was used for inducing aromatase activity. Cell cultures were incubated with cyclic AMP in the absence of FCS for 72 hours prior to assay.

- Cultures were preincubated in the absence of aromatase inhibitors (72 hrs) and assayed in its presence of various aromatase inhibitors – letrozole, anastrozole, aminoglutethimide, fadrozole, formestane and exemestane. This assay procedure parallels in situ studies in patients whom assays are preformed in the presence of the drug.



- Cultures were preincubated in the presence of various aromatase inhibitors – letrozole, anastrozole, aminoglutethimide, fadrozole, formestane and exemestane (72 hrs) and assayed in its absence. This parallels the ex vivo study where the tumour is removed from the presence of the drug.



## RESULTS

Results are expressed as mean and are the result of atleast three separate experiments.

SPSS version 14.0.2 (SPSS, Chicago, Illinois, USA) was used for all statistical analysis.

Data was analysed using the Mann Whitney U test.  $P < 0.050$  was considered statistically significant.

## **ISOLATION OF NUCLEIC ACID**

### **2.3a Isolation of total RNA from primary cultured cells**

Total RNA was extracted from cultured cells using a modification of the acid guanidium phenol chloroform (AGPC) method of Chocczynski and Sacchi [1987]. In order to minimise RNase contamination of the RNA preparation all solutions were prepared using RNase free glassware, water, plasticware and chemicals. Wherever possible the solutions were treated with 0.1% DEPC (diethyl pyrocarbonate) for 24 hours at room temperature and subsequently autoclaved for 20 mins at 15lb/sy on a liquid cycle.

### **2.3b Isolation of total RNA from snap frozen tissue**

Breast tumour tissue obtained before and after treatment with letrozole was promptly washed in Dulbecco 'A' PBS and snap frozen in liquid nitrogen. The frozen tissue was stored at  $-70^{\circ}\text{C}$ . The frozen tissue was disaggregated using a mikro dismembranator fitted with a 7ml Teflon shaking flask and accompanying stainless steel grinding ball, both of which were prechilled in liquid nitrogen. The dismembranator was operated on full power for 20 seconds and the resulting tissue powder scraped into 5ml of solution using a prechilled micro-spatula. The stainless steel grinding ball was also placed into the aliquot of solution, since a significant amount of the powdered tissue adhered to the ball. The RNA preparation was continued as outlined below.

### **2.3c Quantitation**

A 5 $\mu$ l of the RNA solution was diluted in nuclease free water (2.995ml) and the concentration and the purity of the preparation determined spectrophotometrically. In order to quantify the amount of RNA in the sample readings were taken at 260nm and 280nm. An OD<sub>260</sub> of 1 corresponds to an RNA concentration of 40 $\mu$ g of RNA/ml. The ratio between readings taken at 260nm and 280nm (OD<sub>260</sub>/ OD<sub>280</sub>) provides an estimate of the purity of the sample. A ratio of 2 corresponds to a pure sample and if the ratio was less than 1.7 the sample was re-extracted with phenol chloroform.

### **2.3d Extraction of RNA**

45 $\mu$ l of phenol: chloroform (1:1) was added to the RNA solution and the suspension vortexed and spun at 13,000 rpm for 5 mins (4°C). The aqueous phase was transferred to a fresh eppendorf tube and mixed with 45 $\mu$ l of chloroform: isoamylalcohol (49:1) prior to a further centrifugation at 13,000 rpm for 5 mins (4°C). The aqueous phase was removed and RNA precipitated by the addition of 3 volumes of ethanol and 3M sodium acetate to 0.3M final concentration. The precipitated RNA was pelleted and resuspended as previously described. The concentration of RNA solution could then be reassessed spectrophotometrically as before.

### **2.3e RNA check gel**

The 2% agarose gel was prepared by melting 1 g of agarose NA in 75 ml of 1X TBE using a microwave oven. The molten agarose was cooled to approximately 40°C before

adding 1µl of 10mg/ml ethidium bromide. The agarose was then poured into a level tray containing a comb of well dimension 5.5 x 1.5 mm 16 well comb. The gel was run in 800 ml of 0.5 X TBE in midi-gel apparatus.

5µl of sample and 1µl of loading buffer (6XM) could be loaded per well alongside Lambda DNA restricted by EcoRI and Hind III. Electrophoresis was carried out at 100 volts for 45 minutes. Then the gel was examined and photographed under ultra violet transillumination. The RNA sample was considered good if 8S and 24S bands were the only clear visible major bands on electrophoresis. Only RNA samples that were good both by spectrophotometry and by electrophoresis have been taken for reverse transcription (RT).

### **2.3f Reverse transcription of total RNA**

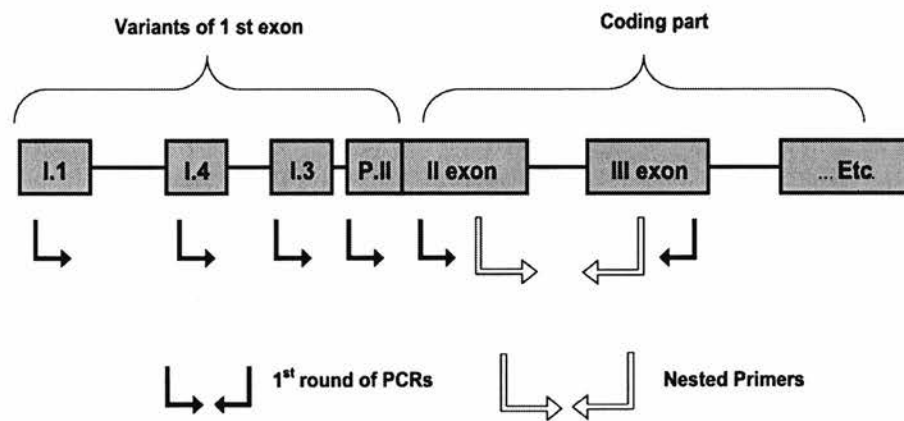
The protocol for first strand synthesis of cDNA from samples of total RNA is a modification of that described in the Promega protocol. A volume of total RNA solution corresponding to 1.5µg total RNA was aliquoted into a 500µl test tube. The tube was then heated to 85°C for 5 minutes and cooled on ice for 3 minutes. It was pulsed in a microcentrifuge and the RNA mix was added to the solution. The tube was vortexed, pulsed in a microcentrifuge and then incubated at 42°C for 60 minutes. Then the reverse transcription mixture was heated to 90°C for 5 minutes and followed by a 5 minutes incubation on ice. Potency of reverse transcription (RT) was verified by PCR with primers to GAPDH gene.

### 2.3g Aromatase gene

Expression of the coding part of aromatase mRNA was verified examining the region across exons 2 and 3 (**fig 3**). The four most common variants of the untranslated and alternatively spliced first exon were also studied (I.1, I.4, I.3 and PII variants). In all instances, a nested design of PCR was used; after a first round of PCR, 1/10 of PCR products were taken for the second PCR with nested primers. Primers sequences and cycling conditions are in **table 7 and 8**. PCR products were detected following electrophoresis in 2% agarose gel and staining with ethidium bromide.



Fig 3: DESIGN OF NESTED PCR



**Table – 7: Primer sequences and cycling conditions**

<b>Exon</b>	<b>Primer</b>	<b>Notes</b>
<b>Sequences</b>		
I.1 (1a)	CTG GAG GGC TGA ACA CTG GG	Harada et al (1993)
I.4 (1b)	GAC CAA CTG GAG CCT GAC A	
I.3 (1c)	CCT TGT TTT GAC TTG TAA CCA	
P.II (1d)	AAC AGG AGC TAT AGA TGA AC	
II for 1st round	GTC AAG GAA CAC AAG ATG GT	
II nested	ATC GTG CCT GAA GCC ATG CC	Based on the Sequences of II and III exons published in the Gene Bank
III nested	TTG TAG TAG TTG CAG GCA CT	
III for 1st round	ATA ATG AGT GTT TCC TCT CC	

Table -8: **PCR conditions**

Step	PCR-mix	Cycling
<b>1st round of PCR</b>	2 mM MgCl <sub>2</sub> , 0.2 mM dNTP, 1 ×PCR buffer and Taq 25 mU/μl (Promega) 0.5 μM 1 <sup>st</sup> round primers Total volume of 25 μl	Initial denaturation: 95°C 3 min; 40 cycles: 95° C 30s, 50°C 45s, 72°C 30s Final elongation: 72°C 3 min
<b>2nd round of PCR</b>	2 mM MgCl <sub>2</sub> , 0.2 mM dNTP, 1 ×PCR buffer and Taq 25 mU/μl (Promega) 0.5 μM 2 <sup>nd</sup> round primers, 2.5μl of 1 <sup>st</sup> round PCR products Total volume of 25 μl	Initial denaturation: 95°C 3 min; 35 cycles: 95° C 30s, 57°C 30s, 72°C 30s Final elongation: 72°C 3 min

## **2.3h Agarose gel electrophoresis of PCR products**

The PCR products were analysed by electrophoresis in 2% agarose gels containing 1X trisborate EDTA buffer (TBE).

### **Preparation of agarose gel**

The 2% agarose gel was prepared by melting 1g of agarose in 75 ml of 1X TBE using a microwave oven. The molten agarose was cooled to approximately 40°C before adding 1µl of 10mg/ml ethidium bromide. The agarose was then poured into a level tray containing a comb of well dimension 5.5 x 1.5 mm 16 well comb.

### **Electrophoresis of samples in agarose gel**

The gel was run in 800 ml of 0.5 X TBE in midi-gel apparatus. Bluescript and lambda DNA were used as molecular weight markers. 18µl of each PCR reaction with 2µl of DNA loading buffer could be loaded per well alongside. Electrophoresis was performed for 4 hours at 50 volts after which the gel was examined and photographed under UV transillumination.

## **RESULTS**

### **1. Validation experiments**

- 1.1 Validation of aromatase assay
- 1.2 Effect of letrozole on aromatase activity in placenta
- 1.3 Letrozole in assay phase
- 1.4 Invitro test system for measuring aromatase inhibition

### **2. Effects of aromatase inhibitors on aromatase in cultured fibroblasts [in the presence of Dexamethasone]**

- 2.1 Incubation of fibroblast culture with aromatase inhibitors
- 2.2 Preincubation of fibroblasts culture with letrozole
- 2.3 Preincubation of fibroblasts culture with anastrozole, aminoglutethimide and fadrozole
- 2.4 Preincubation with type I aromatase inhibitor - formestane

### **3. Effects of aromatase inhibitors on cultured fibroblasts [in the presence of Cyclic AMP]**

- 3.1 Preincubation of cultured fibroblasts with letrozole and anastrozole
- 3.2 Preincubation of fibroblasts culture with fadrozole and aminoglutethimide
- 3.3 Preincubation of fibroblasts culture with type I aromatase inhibitors
- 3.4 Incubation of fibroblasts in the presence aromatase inhibitors

### **4. Preincubation of fibroblast culture with Androstenedione**

**5. Effect of aromatase inhibitors on aromatase activity with time**

**6. Exvivo studies**

6.1 Treatment with letrozole

6.2 Treatment with anastrozole

6.3 Treatment with exemestane

**7. Treatment of breast adipose tissue with Letrozole - PCR**

## RESULTS

### 1. VALIDATION EXPERIMENTS

#### 1.1 Validation of aromatase assay

Aromatase activity was measured by tritium release assay method. The determination of aromatase activity was based on the measurement of ( $^3\text{H}$ ) water after incubation with ( $1\beta^3\text{H}$ )  $\Delta$  4 androstenedione. Mammary fibroblasts were used as test system and the radioactivity in the aqueous phase was measured after charcoal extraction as described before. Supernatant (1ml) from one tube was transferred into a background glass counting vial (A) containing liquid scintillator and the supernatant from the other tube was transferred into a counting vial (B). The second aliquot (B) was evaporated to dryness under a continuous stream of nitrogen to eliminate any tritiated aqueous products. All vials were vortex mixed and their radioactivity measured. The results are shown in **table –9**.

Blank incubations are included in each experiment to assess background radioactivity. The blanks, which contained only media but no cells, demonstrated a very low radioactivity confirming that the background activity was very low. The fibroblasts (control) displayed significant aromatase activity as high as 9267 dpm. This activity was profoundly inhibited by the aromatase inhibitor letrozole (100nm) by almost 97% demonstrating that letrozole is a potent aromatase inhibitor.

Evaporation of duplicate aliquots (tube B) - blank, control and with letrozole contained only very low levels of radioactivity. The low counts measured following evaporation confirms that  $^3\text{H}$  released into water was only measured and the aqueous phase was not

contaminated by tritiated steroids. This experiment confirmed the validity of the methodology used.

Tritium release assay quantifies the release of tritium from the  $1\beta$  position of the substrate into the aqueous phase of the reaction mixture. Although the tritium atoms are located in both  $\alpha$  (20%) and  $\beta$  (80%) positions of the substrate, only the tritium atom in the  $\beta$  position is incorporated into water during the aromatisation reaction. Thus, the conversion rate can be determined by the isolation and quantification of the tritiated water.



**Table-9: Validation of aromatase assay**

The table represents the mean dpm (of atleast three experiments) before and after evaporation.

Sample	Before Evaporation	After Evaporation	A-B
	A	B	
Blanks	77	55	22
Control	9267	458.7	8808
With letrozole	96	10.5	84.5

Blanks – contains no cells

## 1.2 Effect of letrozole on aromatase activity in placenta

The potency of aromatase inhibitor letrozole (100nm) to inhibit aromatase activity was tested using the placental system. Placenta was obtained and processed as previously described. Aromatase activity was determined by tritium release assay.

The results are shown in **table 10**. 97% of aromatase activity was inhibited when placental microsomes were incubated in the presence of letrozole. Significant aromatase activity was observed with placenta. Letrozole consistently inhibited aromatase activity in all the experiments carried out (atleast three). This has again demonstrated that the new generation aromatase inhibitors such as letrozole are potent inhibitors of aromatase activity.

**Table – 10: Effect of letrozole on aromatase activity in placenta**

Represents the dpm of three experiments conducted separately.

<b>Expt No</b>	<b>Blanks</b>	<b>Placenta</b>	<b>with letrozole</b>	<b>% Inhibition</b>
1.	81	12538	367	97
2.	75	11268	397	97
3.	71	12641	348	97

### 1.3 Letrozole in assay phase

Fibroblasts were preincubated with dexamethasone ( $1\mu\text{M}$ ) for 18 hours and 15% FCS and letrozole (100nm) was added during the assay phase.

Interestingly the fibroblasts demonstrated significant aromatase activity. 97% of aromatase activity was markedly inhibited by letrozole as seen in **table-11**. Letrozole has thus consistently inhibited aromatase activity by 97% irrespective of using placental microsomes or fibroblasts.

**Table-11: Aromatase activity with letrozole (100nm) in the assay phase**

Experiment No	Sample	DPM	% of Inhibition
1.	Control	12639	97
	Letrozole	279	
2.	Control	9267	97
	Letrozole	209	
3.	Control	11308	97
	Letrozole	265	

## 1.4 Invitro test system for measuring aromatase inhibition

Cultured mammary fibroblasts, placenta and breast cancer homogenates are useful to assess the ability of aromatase inhibition by aromatase inhibitors. The inhibitory effects IC<sub>50</sub> (50% inhibitory concentration) of aminoglutethimide, letrozole, anastrozole, exemestane and formestane are summarised in **table - 12 and 13 a & b**.

Aminoglutethimide, letrozole, anastrozole, exemestane and formestane inhibited aromatase activity in a dose related manner in each system tested; but the newer drugs were magnitudes of order more potent than aminoglutethimide. Thus, micromolar concentrations were required for aminoglutethimide while only nanomolar concentrations were needed with letrozole, anastrozole, exemestane and formestane. The newer type II inhibitors (letrozole, anastrozole) were more potent than type I inhibitors (exemestane and formestane). Letrozole and exemestane are more potent in whole cell cultures than in disrupted cell preparations. Amongst the type II inhibitors letrozole is the most potent and exemestane is more potent than the other type I agent formestane.

**Table 12: Relative invitro potency of aromatase inhibitors as determined using mammary fibroblast cultures as a test system**

<u>Mammary fibroblast cultures</u>		
<b>Aromatase Inhibitor</b>	<b>IC<sub>50</sub> [nM]</b>	<b>Relative potency [compared to aminoglutethimide]</b>
Aminoglutethimide	8000	1
Anastrozole	14	570
Letrozole	0.8	10,000
Formestane	45	180
Exemestane	5	1600

**Table 13 a: Relative invitro potency of aromatase inhibitors as determined using placental microsomes as a test system**

Placental Microsomes		
Aromatase Inhibitor	IC <sub>50</sub> [nM]	Relative potency [compared to aminoglutethimide]
Aminoglutethimide	3000	1
Anastrozole	12	250
Letrozole	12	250
Exemestane	50	60
Formestane	50	60



**Table 13 b: Relative invitro potency of aromatase inhibitors as determined using breast cancer homogenates as a test system**

<b>Aromatase Inhibitor</b>	<b>Breast cancer homogenates</b>	
	<b>IC<sub>50</sub> [nM]</b>	<b>Relative potency [compared to aminoglutethimide]</b>
Aminoglutethimide	4500	1
Anastrozole	10	450
Letrozole	2.5	1800
Exemestane	15	300
Formestane	30	150

## DISCUSSION

In this chapter, the methodology and the different types of test system used are discussed.

The tritium release assay was used to measure aromatase activity in this study. Tritium release assay was originally described by Thompson and Siiteri and modified by Ackerman et al. It is based on the measuring the amount of  $3\text{H}$ -water released upon enzymatic conversion of radiolabeled androstenedione during aromatisation of androstenedione or testosterone to oestrone. However, the assay requires the use of radioactive materials and specialised equipment for radiometric measurement [Stresser DM]. Tritiated water assay quantifies the release of tritium from the  $1\beta$  position of the substrate into the aqueous phase of the reaction mixture. Aromatase activity is quantified by the stereo specific loss of  $1\beta$   $^3\text{H}$  of the substrate into the aqueous phase of the reaction mixture during aromatisation.

Tritiated substrates in which the radiolabel is present at both  $1\beta$  and  $2\beta$  positions are commonly available and these lead to higher blank values owing to the ability of the hydrogen at the 2 position to exchange albeit at a low rate with water [Siiteri PK, 1982].  $1\beta$   $^3\text{H}$  labelled substrates are utilised in this study to increase the specificity of the assay and the background counts, especially when tissue samples are used in which enzyme activity is so low. Blank incubations are included in each experiment to assess background radioactivity. Only low levels of radioactivity were measured in the blanks (background) in the experiments conducted. The background activity was assessed by

using blanks (using media only) and the experiments were always performed in duplicates, thus eliminating experimental bias in the methodology.

The methodology was verified by the low counts measured (following evaporation of  $^3\text{H}_2\text{O}$ ) in the validity experiments, which confirms that  $^3\text{H}$  released into water was only measured and the aqueous phase was not contaminated by tritiated steroids.

There are different types of methods by which the biochemical efficacy of an aromatase inhibitor may be assessed: invitro measurements, evaluation in animal models and invivo assessment of endocrine efficacy in humans [James VHT, 1989]. The latter two models were beyond the scope of this thesis.

An invitro model system was used in this study to test the potency of aromatase inhibitors and test systems such as placenta, breast cancer homogenate, adipose tissue and cultured fibroblasts were used in this study. The reason for using different test systems including its advantages and disadvantages are addressed.

There are numerous test systems, which can be used to assess the ability of different agents to inhibit aromatase activity. These screens include placental microsomes, particulate tumour fraction and whole cell system. The most widely used test system for aromatase inhibition is placental microsomes; although universally employed, they do not reflect the low levels of aromatase activity seen in peripheral tissues [Stresser DM]. In addition, if antiaromatase agents are to be used in postmenopausal breast cancer patients, it seems appropriate to screen these drugs against peripheral tissues, which are the primary sites of oestrogen biosynthesis in postmenopausal women. About 70% of breast cancers, when incubated as particulate fraction (invitro) display aromatase activity [Miller WR, 2000] and these can be used as a screen. Therefore, it is more appropriate to

screen aromatase inhibitors against peripheral tissues. Finally, while disrupted cell preparations are useful test systems, they do not account for other factors such as cellular drug uptake; therefore it is advisable to include whole cell system such as cultured fibroblasts in any screen. Thus whole cell system such as placenta, breast cancer homogenate and cultured fibroblasts were used in this study to determine the effects of aromatase inhibitors.

The reason for using fibroblast cell culture from mammary adipose tissue is discussed. Aromatase activity mainly occurs in stromal cells that surround adipocytes and these stromal cells grow in cell culture as fibroblasts [Price T]. Adipose tissue is the major site of oestrogen biosynthesis in postmenopausal women, with the local production of oestrogen in breast adipose tissue implicated in the development of breast cancer [Rubin GL]. This is based on the following evidence: (i) fat is able to aromatise androgens (ii) fat is a major component of body mass [Forbes GB] (iii) fat is the main reservoir for steroids [Deslypere JP] and (iv) degree of obesity relates to the level of circulating oestrogens and incidence of oestrogen-dependent conditions [MacDonald PC]. The large mass in the body fat is expected to be the main contributors to total peripheral oestrogen formation mainly in postmenopausal women [Forbes GB]. Microsomal fractions or cultured fibroblasts (derived from mammary adipose tissue) yields small quantities of oestrogen if incubated with androgen precursors and this would release picromolar quantities of oestrogen which would be sufficient to mediate oestrogen mediated events if this potential was realised in situ (although it has low levels of conversion). Therefore mammary fibroblast cell culture model system was more appropriate to be used in this study.

A variety of tissue preparations have been used to measure adipose tissue aromatase activity (invitro). These include tissue slices, minces, cell suspension or culture, homogenates and various sub cellular fractions. The method described by Nimod and Ryan utilized the soluble extract of tissue homogenates and has subsequently been used successfully by a number of groups. In this study fibroblast, cell culture prepared from breast adipose tissue was mainly used.

It should be noted that all the tissues were processed similarly and no variation in materials or methods were introduced. All experiments were conducted in primary cell cultures without passaging, as both basal and induced aromatase activity were maximal in primary and early passage cultures and aromatase activity markedly decreased with increasing passage [Miller WR, 1993]. These results whilst not definitive could be consistent with local secretion of factors by tumours, which are capable of transiently influencing aromatase activity in adipose tissue.

Before embarking upon studies investigating the effects of aromatase inhibitors on aromatase activity, a series of control experiments were undertaken. The consistency in reproducibility of the methodology was initially ascertained by conducting several preliminary experiments using cultured fibroblasts and placenta. Adipose tissue was used in the majority of the experiments. In some of the preliminary experiments placenta was used since they exhibit higher aromatase activity than adipose tissue and therefore require shorter incubation times. Significant aromatase activity could be demonstrated in both fibroblasts and placenta. The effect of aromatase inhibitors such as letrozole was tested using placenta and fibroblasts culture. 97% of aromatase activity was inhibited by letrozole demonstrating its remarkable potency.

Aromatase inhibitors also exhibited different potency in the different test systems. The sensitivity to individual inhibitors was similar to that previously reported in placental microsomes [di Salle R; Plourde PV, 1994]. In breast cancer homogenates, the two type II inhibitors (letrozole, anastrozole) were more potent than the type I drugs (exemestane, formestane) [Miller WR, 1997]. It was also observed that letrozole and exemestane seemed to have greater potency in cultured fibroblasts. This could relate to the differences between disrupted and whole-cell preparations or between malignant and nonmalignant tissues. Interestingly, letrozole seems to have increased potency in whole cell systems and it has been postulated that the drug may accumulate to a greater extent in such systems [Bhatnagar AS, 1999].

## **2. EFFECTS OF AROMATASE INHIBITORS ON AROMATASE IN CULTURED FIBROBLASTS [in the presence of Dexamethasone]**

### **2.1 Incubation of fibroblast culture with aromatase inhibitors**

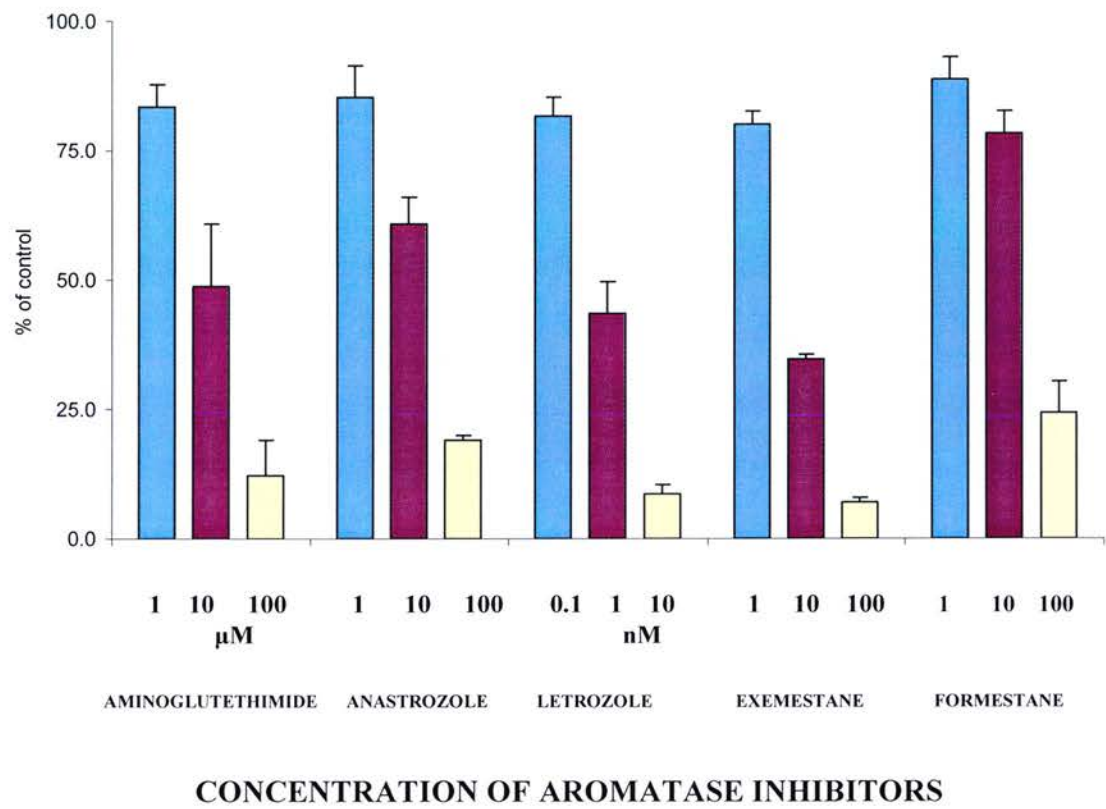
Fibroblast cultures were incubated in the presence of dexamethasone for 18 hours and then assayed for aromatase activity in the presence of various inhibitors – aminoglutethimide, letrozole, anastrozole, exemestane and formestane. The results are shown in **fig - 3**.

All the drugs produced a dose response curve. Aromatase activity was markedly suppressed at all concentrations studied with both types (I and II) of inhibitors.

The highest inhibitory effect was seen with 100 $\mu$ M of aminoglutethimide, 10nm of letrozole while anastrozole, exemestane and formestane produced maximum inhibitory effect at 100nm concentration ( $p<0.001$ ). Thus all the drugs apart from aminoglutethimide required nanomolar concentrations to produce inhibitory effect, while aminoglutethimide required micromolar concentration. Letrozole was more potent than other type II inhibitors ( $p<0.001$ ) and exemestane was more potent than formestane ( $p=0.039$ ).

**Fig 3: Incubation of fibroblasts cultures with aromatase inhibitors**

Effect on aromatase activity expressed as percentage [%] of control cultures without inhibitors and are mean of at least three separate experiments. The columns represent the means and the bars the standard errors of the means.





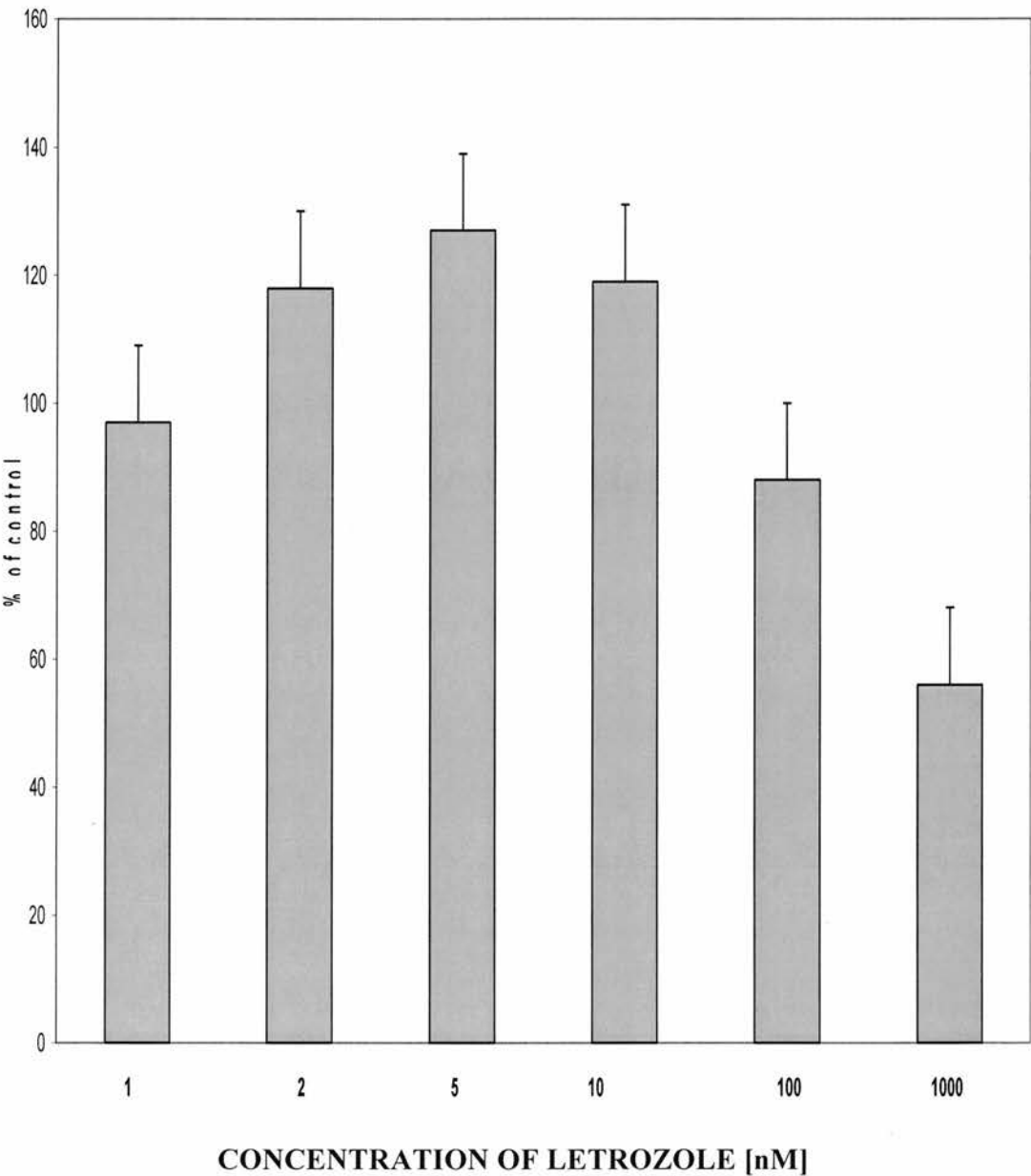
## 2.2 Preincubation of fibroblasts culture with letrozole

Cultures were preincubated with letrozole (1-1000nm) for 18 hours and then assayed for aromatase by incubating with  $1\beta^3\text{H}$   $\Delta$  4 androstenedione for 5 hours in the absence of letrozole. The determination of aromatase activity was based on tritium release assay as previously described. The results are shown in **fig 4a and 4b**.

Preincubation with letrozole produced enhanced activity at lower concentrations (2-20 nm) and this was statistically significant ( $p < 0.005$ ). Inhibition of aromatase activity could be demonstrated at higher concentrations (100 -1000 nm) of letrozole ( $p < 0.0005$ ). Interestingly drug doses between 2 and 20nm consistently produced an increase in aromatase activity. The maximum inductive effect of about 127% was produced with letrozole at a concentration of 5nm.

**Fig 4a: Preincubation of fibroblasts with letrozole**

Effect on aromatase activity expressed as percentage [%] of control cultures without inhibitors and are mean of at least three separate experiments. The columns represent the means and the bars the standard errors of the means.

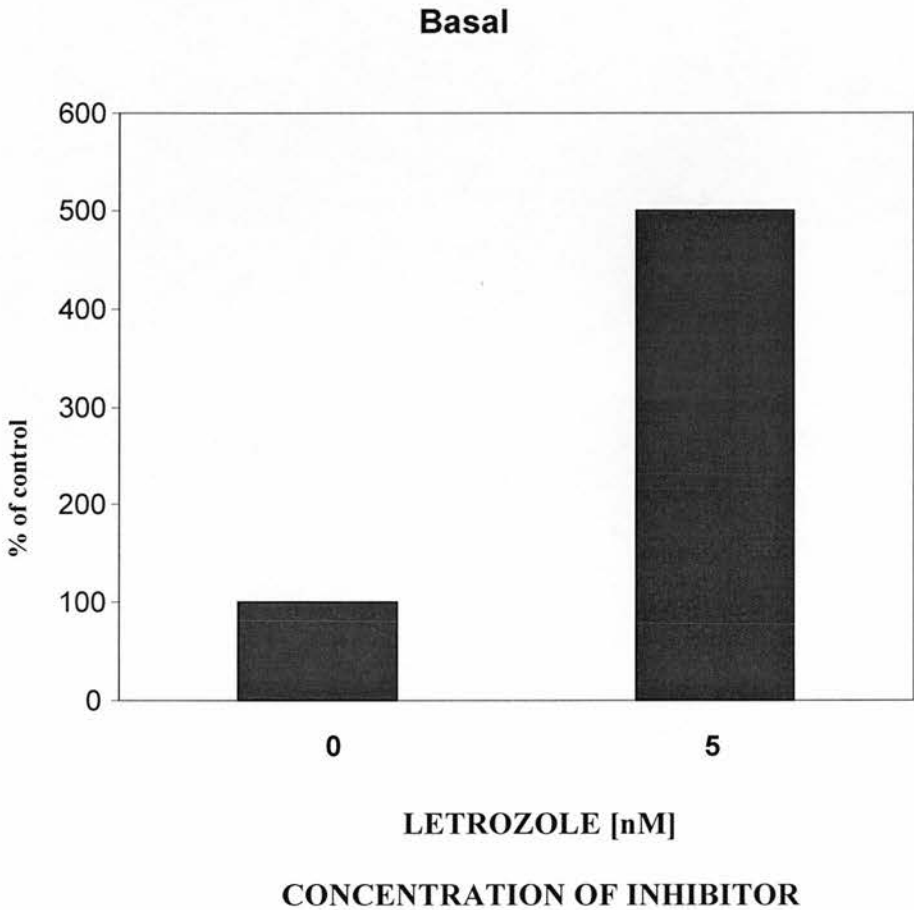


Cultures were preincubated with letrozole (100nm) in the absence of dexamethasone. Tritium release assay was carried out as described before.

**Fig 4b** shows the effects of preincubation with letrozole (in the absence of dexamethasone).

Letrozole produced five-fold increase in basal levels (which were low).

**Fig 4b: Effects of preincubation with Letrozole in the absence of dexamethasone**



## **2.3 Preincubation of fibroblasts culture with anastrozole, aminoglutethimide and fadrozole**

Cultures were preincubated with anastrozole (0.1-1000nm), fadrozole (1-100nm) and aminoglutethimide (1 $\mu$ m -10 $\mu$ m) and then assayed for aromatase activity in the absence of inhibitors as previously described.

Pre-incubation produced enhanced aromatase activity at all concentrations studied with all the three inhibitors - anastrozole, fadrozole and aminoglutethimide. The results are shown in **figure 5**.

Anastrozole produced enhancement of aromatase activity at all concentrations (0.1 - 1000 nm) studied rather than the expected inhibitory effect ( $p < 0.0005$ ). Interestingly, inductive effect could be demonstrated even at 0.1nm ( $p = 0.004$ ). Inductive effect remained almost stable between 1 – 100nm and demonstrated a gradual decline at 1000 nm.

Fadrozole induced aromatase activity at all doses studied with maximum effect seen at 10nm ( $p = 0.008$ ). However with an increase in the concentration (100nm), a gradual decline in the inductive effect was noted ( $p < 0.05$ ).

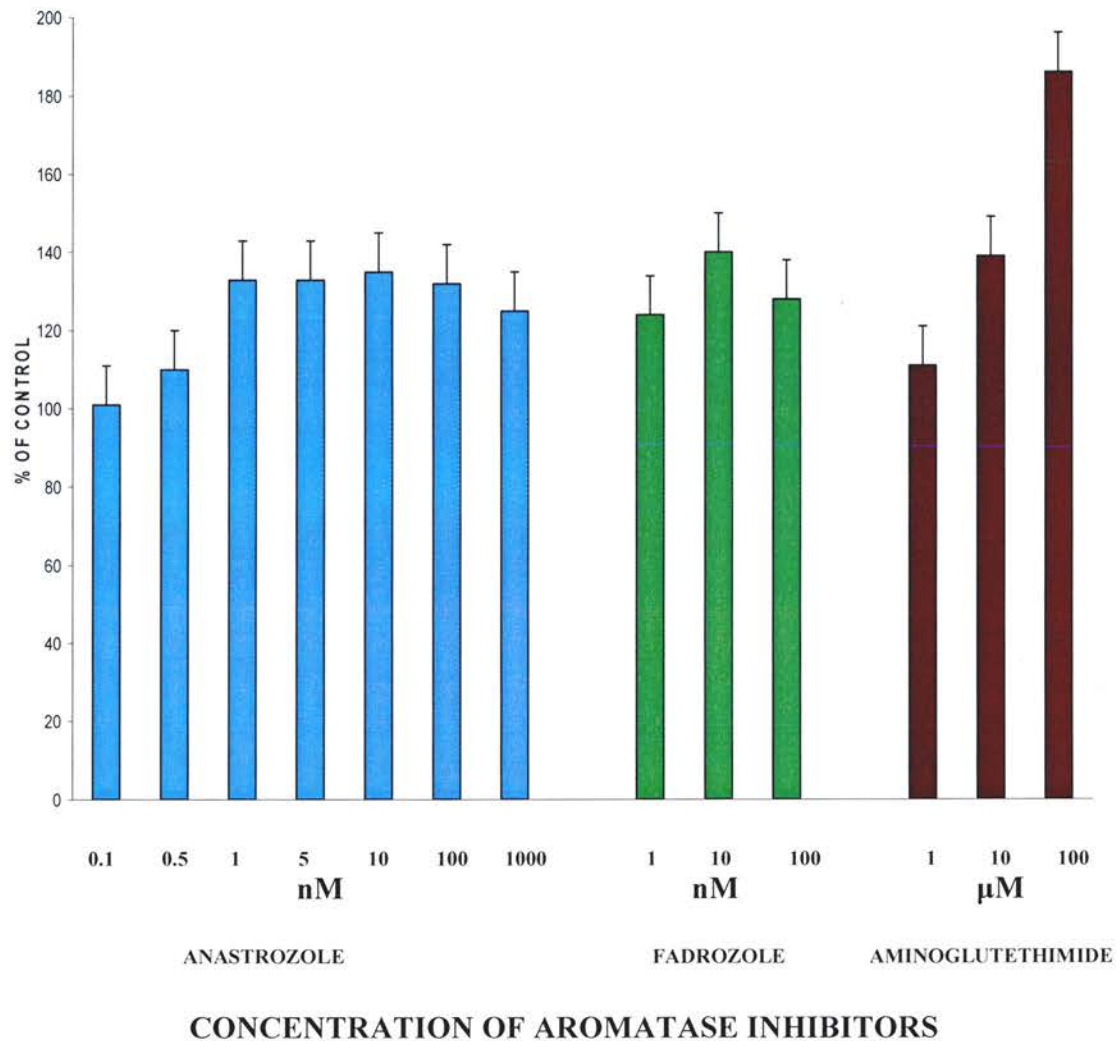
Aminoglutethimide also consistently induced aromatase activity at all concentrations studied ( $p = 0.058$ ). Increase in aromatase activity was seen as concentrations were increased from 100nm to 10 $\mu$ m (120 to 190%). Thus the magnitude of inductive effect increased with increase in the concentrations studied ( $p = 0.095$ ).

Preincubation of cultured fibroblasts with type II inhibitors and assaying for aromatase activity in the absence of inhibitors, have demonstrated a variable paradoxical response. This has been contradictory to the effects of aromatase inhibitors *invivo* situation.

However of all the type II inhibitors letrozole alone has been able to retain part of its inhibitory effect at certain concentrations while the other drugs - anastrozole, fadrozole and aminoglutethimide have all failed to realise their full inhibitory potential. This may have important clinical implication, which will be discussed later.

**Fig 5: Preincubation of fibroblasts with aromatase inhibitors - anastrozole, fadrozole and aminoglutethimide**

Values are presented as a % of control cultures and are mean of three separate experiments. The columns represent the means and the bars represent the standard errors of the means.



## **2.4 Preincubation with type I aromatase inhibitor - formestane**

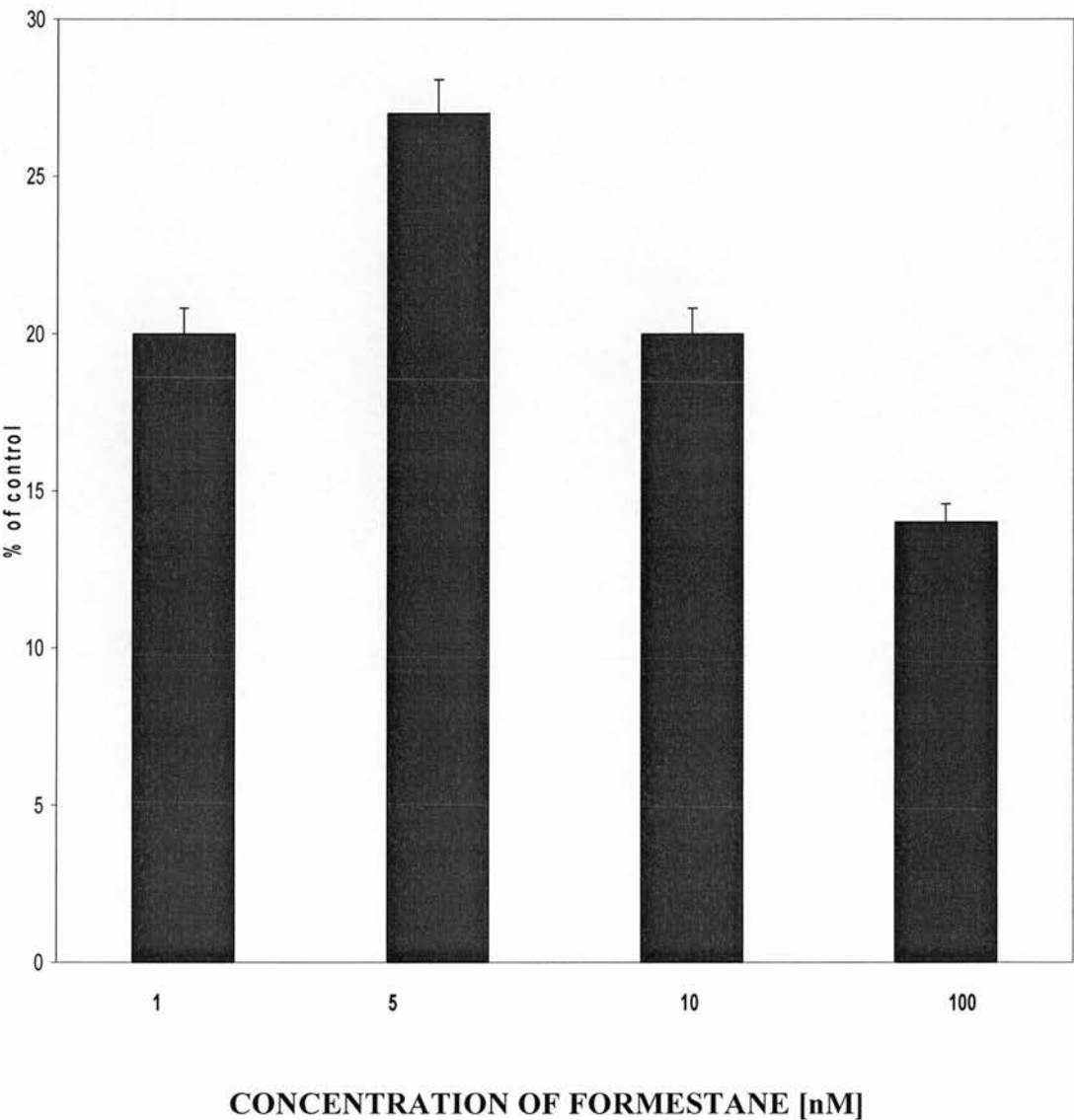
Cultures were preincubated with formestane (1-1000nm) and then assayed for aromatase activity in the absence of the inhibitor. The determination of aromatase activity was based on tritium release assay as previously described. The results are shown in **fig - 6**.

Preincubation with the nonsteroidal inhibitor formestane, markedly inhibited aromatase activity at all concentrations (1nm -1000 nm) studied. The inhibitory effect produced by formestane was statistically significant at all concentrations studied ( $p < 0.0005$ ). However, marked inhibitory effect was noticed with an increase in concentration from 1 to 100nm with 5nm producing maximum inhibitory effect ( $p = 0.001$ ).

Thus formestane demonstrated its inhibitory effect on aromatase activity on preincubation with dexamethasone.

**Fig 6: Preincubation of fibroblasts with formestane**

Values are presented as a % of control cultures and are mean of three separate experiments. The columns represent the means and the bars represent the standard errors of the means





## DISCUSSION

Both type I and type II aromatase inhibitors studied markedly suppressed aromatase activity when assayed in their presence. All the aromatase inhibitors – steroidal (exemestane, formestane) and non steroidal (letrozole, anastrozole, aminoglutethimide and fadrozole) drugs studied produced a dose response with greatest effect observed with the highest concentration studied.

Pre-incubation with type II inhibitors paradoxically enhanced aromatase activity rather than producing the expected inhibitory effect. On the contrary, type I inhibitors markedly and consistently inhibited aromatase activity in all circumstances. Interestingly the inhibitory effect seen with type I inhibitors was greater when pre-incubated with these inhibitors than when the drugs were added during the assay phase itself.

To explore the effects further, fibroblasts cell cultures were preincubated with cyclic AMP and similar experiments were conducted. The results are discussed in the next chapter.

### **3. Effects of aromatase inhibitors on cultured fibroblasts [in the presence of Cyclic AMP]**

#### **3.1 Preincubation of cultured fibroblasts with letrozole and anastrozole**

Cultured fibroblasts were preincubated with letrozole and anastrozole for 72 hours in the presence of cyclic AMP and then assayed for aromatase activity in the absence of inhibitors. The results are as shown in **fig 7**.

Pre-incubation with letrozole and anastrozole resulted in enhanced aromatase activity at all concentrations studied (1-100nm). Letrozole produced inductive effect between doses of 1-100nm ( $p < 0.0005$ ) with maximum inductive effect of 150% seen with 10nm ( $p = 0.002$ ). It must also be noted with letrozole, concentrations above 10nm produce a gradual decline in inductive effect and at a concentration of 1000 nm inhibitory effect was seen ( $p = 0.041$ ).

The aromatase inhibitor anastrozole produced greater inductive effect when compared with letrozole at all concentrations studied. The inductive effect appears to increase with the increase in concentration of anastrozole studied. Anastrozole produced maximum inductive effect of greater than 200% at 100nm ( $p = 0.012$ ). The inductive effect on aromatase activity produced by anastrozole was seen at all concentrations studied (1-1000 nm) and this effect was significant ( $p < 0.0005$ ).

Letrozole produced inductive effect at concentrations of 1-10nm with both dexamethasone (as seen previously) and cyclic AMP and this effect was statistically significant ( $p = 0.02$ ). At 100nm significant inhibitory effect was observed with letrozole

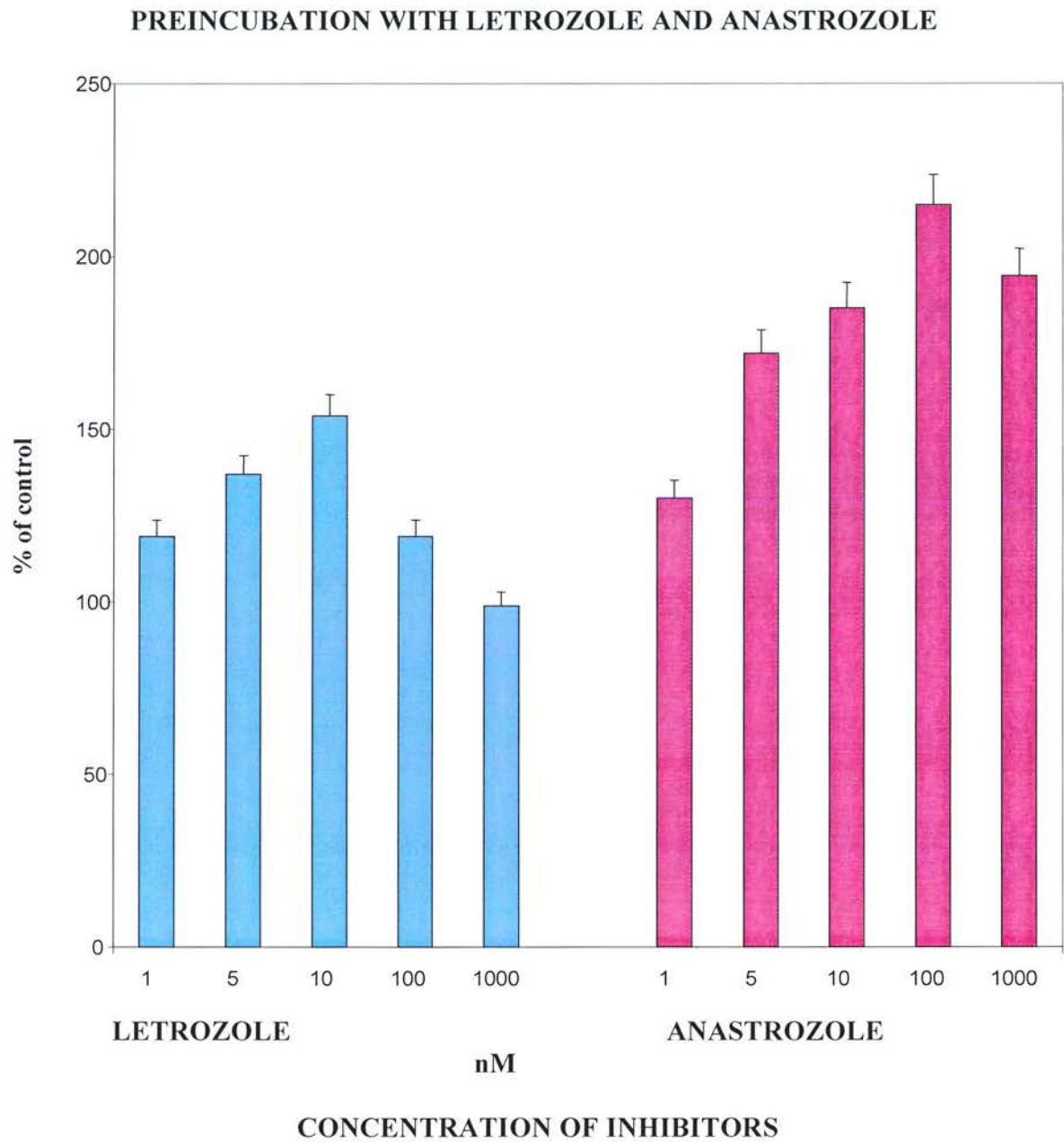
in the presence of dexamethasone while inductive effect was seen with cyclic AMP ( $p=0.02$ ). Letrozole produced marked inhibitory effect at 1000nm in the presence of both dexamethasone and cyclic AMP but the inhibitory effect produced with dexamethasone was highly significant ( $P<0.0005$ ).

Letrozole produced inhibitory effect between 100 - 1000nm in the presence of dexamethasone while a 10-fold increase in concentration is required to produce similar effect in the presence of cyclic AMP. It was also observed that letrozole produced almost a 50% greater inhibitory effect at the same concentration of 1000nm in the presence of dexamethasone (as demonstrated before).

Anastrozole produced inductive effect at all concentrations studied both in the presence of dexamethasone (as seen previously) and cyclic AMP. The inductive effect seen with cyclic AMP was markedly greater than with dexamethasone between concentrations of 5-1000 nm ( $p<0.005$ ). Interestingly at 1nm almost similar inductive effect was produced by anastrozole irrespective of the co-inducer used ( $P=0.65$ ). Thus anastrozole produced inductive effect on preincubation in the presence of both dexamethasone and cyclic AMP.

**Fig 7: Effect on aromatase activity on preincubation of cultured fibroblasts with letrozole and anastrozole**

Expressed as percentage of control systems without inhibitors. The columns represent the means and the bars represent the standard error of the means.



### 3.2 Preincubation with fadrozole and aminoglutethimide

Fibroblasts were pre-incubated with varying concentration of aminoglutethimide ( $\mu\text{M}$ ) and fadrozole ( $\text{nm}$ ) and assay was carried out as previously described. The results are shown in **fig 8**.

Both fadrozole and aminoglutethimide produced an increase in aromatase activity at all concentrations studied.

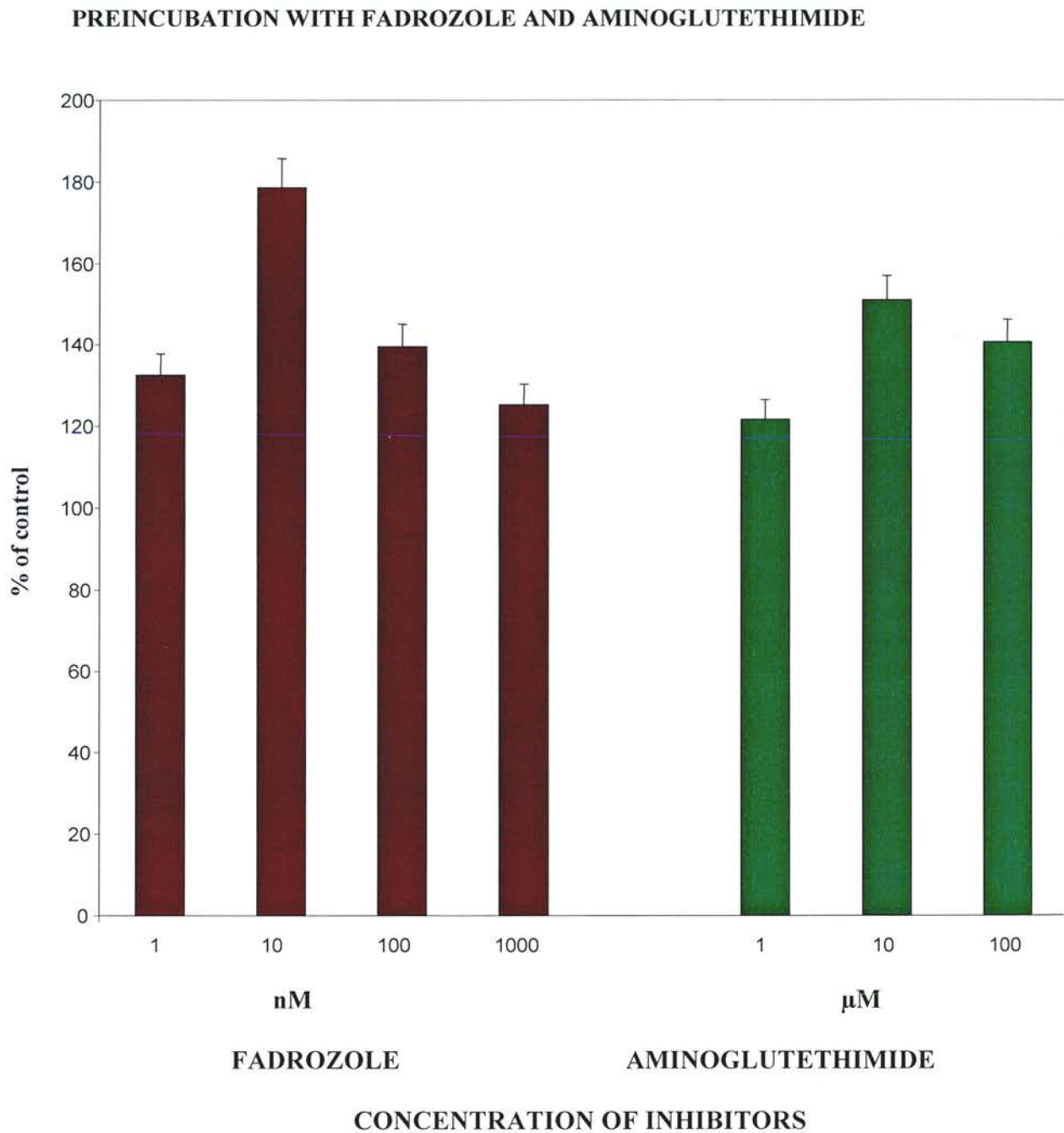
Fadrozole produced a dose response curve. Maximum inductive effect of 179% was seen at 10nm with fadrozole ( $p=0.005$ ). However significant inductive effect was observed with fadrozole in the presence of cyclic AMP ( $P<0.005$ ) than in the presence of dexamethasone (as described before).

Aminoglutethimide produced induction of aromatase activity between doses of 1 to 100  $\mu\text{M}$  concentration and this statistically significant ( $p=0.01$ ). The maximum inductive effect of 150% was seen at a concentration of 10 $\mu\text{M}$  ( $p=0.002$ ). Aminoglutethimide required larger doses (micromolar) concentrations to produce its effects.

The inductive effect produced by aminoglutethimide was markedly greater in the presence of cyclic AMP than in the presence of dexamethasone ( $p=0.005$ ) at all concentrations studied (1-100nm). However aminoglutethimide failed to produce the expected inhibitory effect on preincubation irrespective of the co-inducer used.

**Fig 8: Effect on aromatase activity on preincubation of with fadrazole and aminoglutethimide**

Expressed as percentage of control systems without inhibitors. The columns represent the means and the bars represent the standard error of the means.



### 3.3 Preincubation with type I aromatase inhibitors

The fibroblasts were preincubated with varying concentrations of exemestane and formestane and then assayed for aromatase activity in the absence of the aromatase inhibitors. The results are as seen in **fig 9**.

Aromatase activity was markedly inhibited at all concentrations studied with both exemestane and formestane (1-1000nm). The bell shaped curves were observed with type I inhibitors. Exemestane produced greater inhibitory effect in comparison with formestane ( $p < 0.05$ ) at all concentrations studied (1- 1000nm). This effect may demonstrate the potent effect of exemestane observed in the clinical setting. Maximum inhibitory effect was observed with 10nm of exemestane and 100nm of formestane.

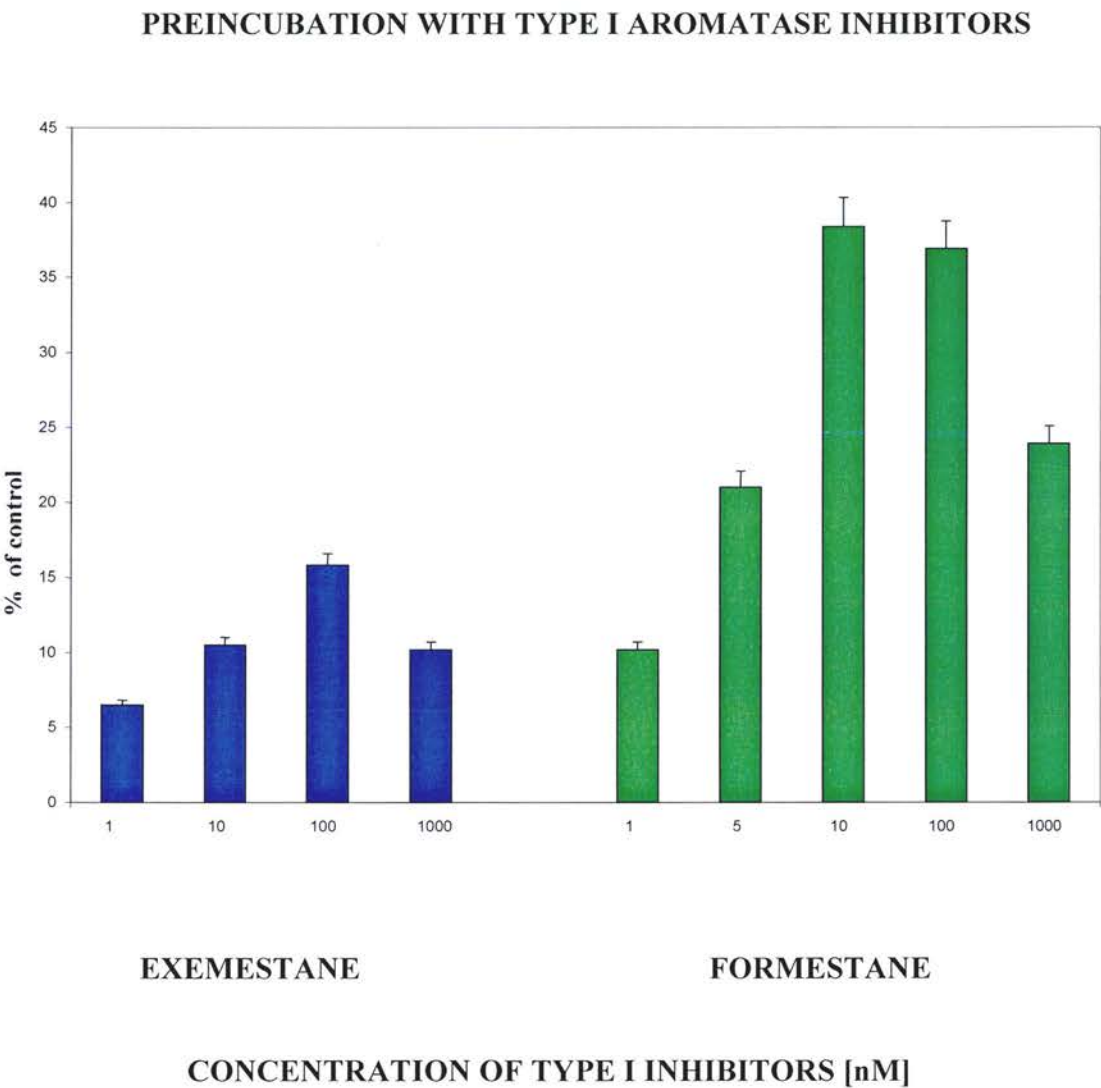
Interestingly, exemestane produced greater inhibitory effect in the presence of dexamethasone (as described before) than in the presence of cyclic AMP ( $p < 0.0005$ ) at all concentrations studied (1-100nm).

Similarly formestane also produced significant inhibitory effect on preincubation with dexamethasone (as described before) than cyclic AMP ( $p = 0.002$ ). The effect observed at 100nm of formestane was statistically significant on preincubation in the presence of both the co-inducers ( $p < 0.0005$ ).

Thus both the type I aromatase inhibitors exemestane and formestane expressed inhibition of aromatase activity in the presence of cyclic AMP.

**Fig 9: Preincubation with type I aromatase inhibitors in the presence of Cyclic AMP**

Effect on aromatase activity expressed as % of control systems without inhibitors. The columns represent the means and the bars represent the standard error of the means of three separate experiments.





### **3.4 Incubation of fibroblasts in the presence of aromatase inhibitors**

Fibroblast cultures were preincubated with cyclic AMP for 72 hours and then assayed in the presence of various inhibitors including letrozole, anastrozole, aminoglutethimide, fadrozole, formestane and exemestane. The results are shown in **fig 10**.

Both type I and type II drugs produced inhibitory effects when assayed for aromatase activity in the presence of aromatase inhibitors. Marked inhibition of aromatase activity was observed at all concentrations with all the aromatase inhibitors studied. However the magnitude of inhibitory effect produced with both type of inhibitors were greater in the presence of dexamethasone (as described before) than in the presence of cyclic AMP and this was statistically significant ( $p < 0.0005$ ).

It must also be noted that the inhibitory effect produced by letrozole was significantly greater than that produced by anastrozole ( $p < 0.01$ ). Aminoglutethimide required larger concentration to produce its effect in comparison to other drugs ( $p = 0.006$ ).

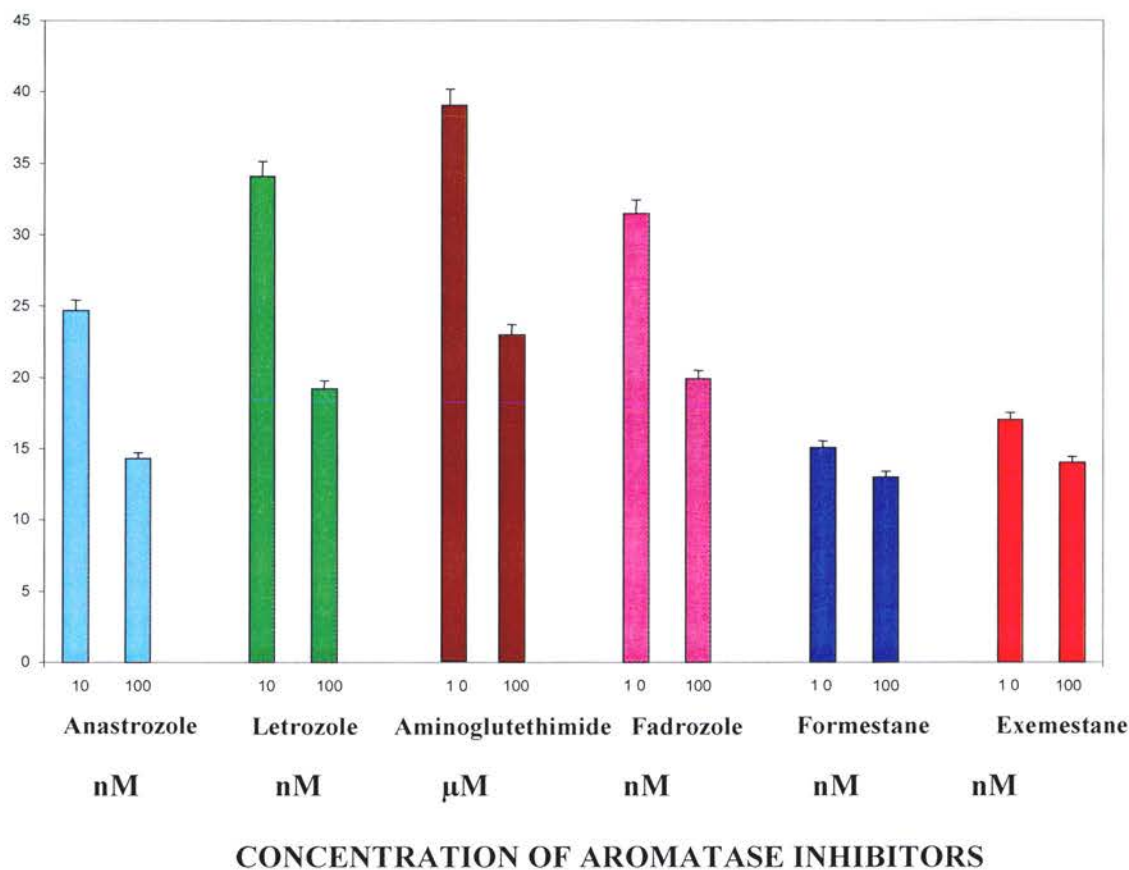
Exemestane and formestane almost produced similar inhibitory effects ( $p < 0.0005$ ). Maximum inhibitory effect was seen at 10nm with both exemestane and formestane ( $p < 0.0005$ ).

Thus all the aromatase inhibitors demonstrated their full inhibitory potential when incubated in the presence of inhibitors irrespective of the co-inducers used.

**Fig 10: Effect on aromatase activity on incubation with various aromatase inhibitors**

Expressed as percentage of control systems. The columns represent the means and the bars represent the standard error of the means.

**AROMATASE ASSAY IN THE PRESENCE OF AROMATASE INHIBITORS**



## DISCUSSION

Aromatase activity was markedly inhibited by both steroidal inhibitors – exemestane and formestane and nonsteroidal aromatase inhibitors – letrozole, anastrozole, fadrozole and aminoglutethimide when assayed in their presence.

Thus similar inhibitory effect was observed with both type I and type II inhibitors irrespective of the inducers used in the study. However the magnitude of inhibition was greater in the presence of dexamethasone than in the presence of cyclic AMP. This may have implication in the clinical setting, which shall be addressed in the discussion chapter.

However the type II aromatase inhibitors failed to realise their full inhibitory effect and produced induction of aromatase activity on preincubation. Letrozole alone produced partial inhibitory effects only at certain concentration (1000nm). Anastrozole produced a greater induction of aromatase activity in the presence of cyclic AMP than in the presence of dexamethasone. The older generation of aromatase inhibitor aminoglutethimide continued to exhibit similar effects but required larger doses in comparison to newer group of drugs.

The paradoxical inductive effect on preincubation was consistently observed irrespective of the co-inducers used with type II inhibitors. This has clearly demonstrated that both the co-inducers - dexamethasone and cyclic AMP has probably no influence on the mechanism causing such paradoxical effects.

Interestingly with type I aromatase inhibitors marked inhibition of aromatase activity was observed on preincubation in the presence of both the co-inducers. Thus the type I inhibitors never failed to exert the expected inhibitory effect in all the experimental setting studied. This may have implication in the sequence of use of aromatase inhibitors, which shall be addressed later in the discussion chapter.

#### 4. Preincubation of fibroblast culture with androstenedione

Fibroblast cultures were preincubated with androstenedione (10  $\mu$ l: 1 $\mu$ ci, substrate) in the presence of aromatase inhibitors (letrozole and formestane) and assayed in the absence of inhibitors. The results are shown in **table - 14**.

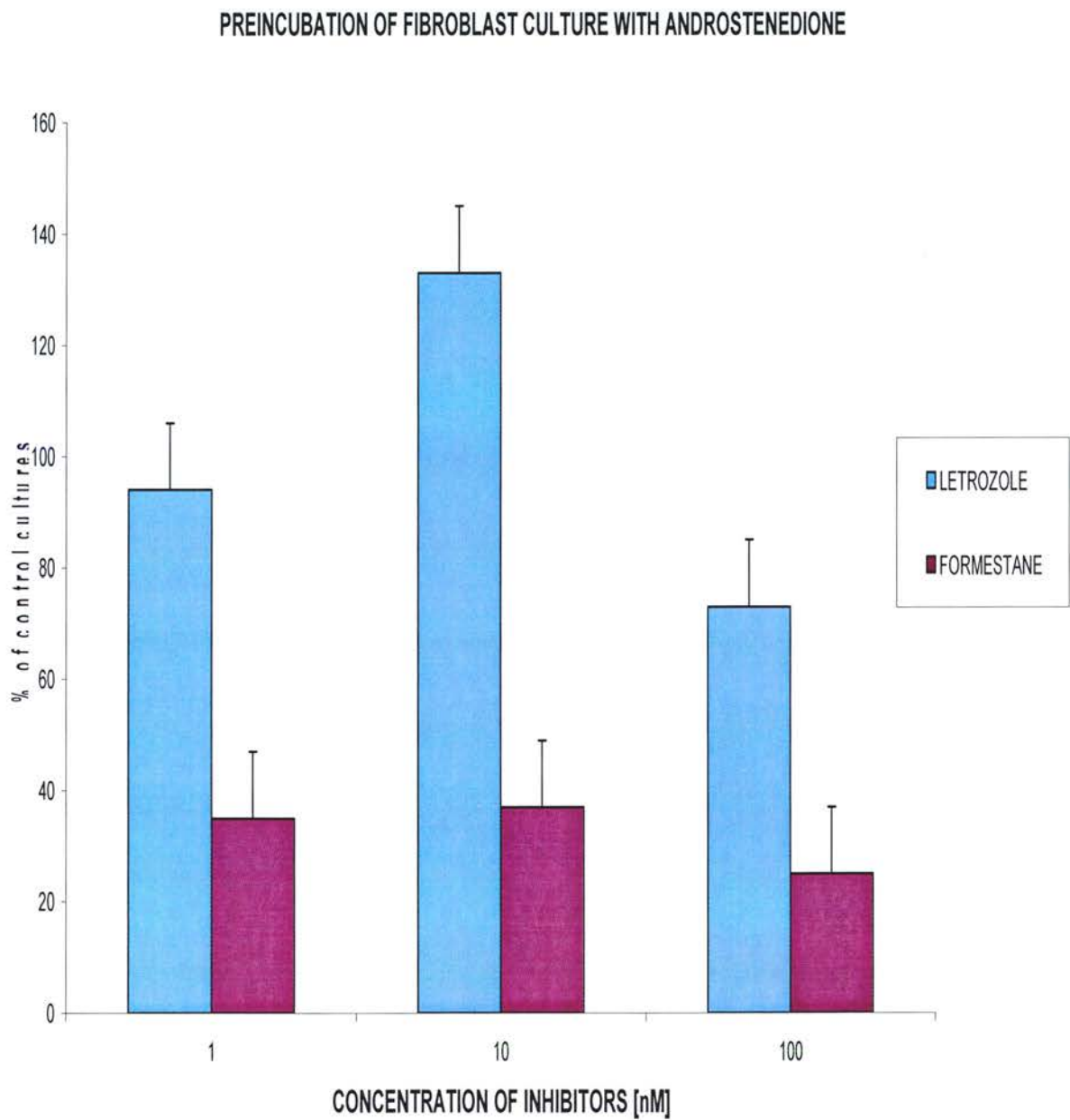
It was observed that letrozole produced a paradoxical induction of aromatase activity at 10nm. However it continued to produce inhibitory effect at certain concentrations – 1nm and 100nm. The response produced by letrozole in the presence of the substrate is similar to the effect produced in the presence of inducers (as described earlier).

On the contrary the type I inhibitor formestane continued to demonstrate marked inhibitory effect even in the presence of androstenedione at all concentrations studied. Formestane demonstrated maximum inhibitory effect at 10nm. The magnitude of inhibitory effect produced by formestane is similar to the response obtained in the presence of inducers - dexamethasone and cyclic AMP (as described earlier). This has demonstrated that the type I inhibitors consistently inhibit aromatase activity in all circumstances.

Thus the addition of substrate did not influence the paradoxical stimulatory effect observed invitro with letrozole at certain concentrations (10nm) ( $p=0.038$ ) and the inhibitory effect observed with formestane ( $p< 0.05$ ) at all concentrations. This may have implications when the aromatase inhibitors are used in the clinical setting and shall be addressed later in the discussion chapter.

**Fig 11: Preincubation of fibroblasts culture with androstenedione**

Values are represented as % of control cultures in the absence of inhibitors. The columns represent the means and the bars represent the standard error of the means of three separate experiments.



## **5. Effect of aromatase inhibitors on aromatase activity with time**

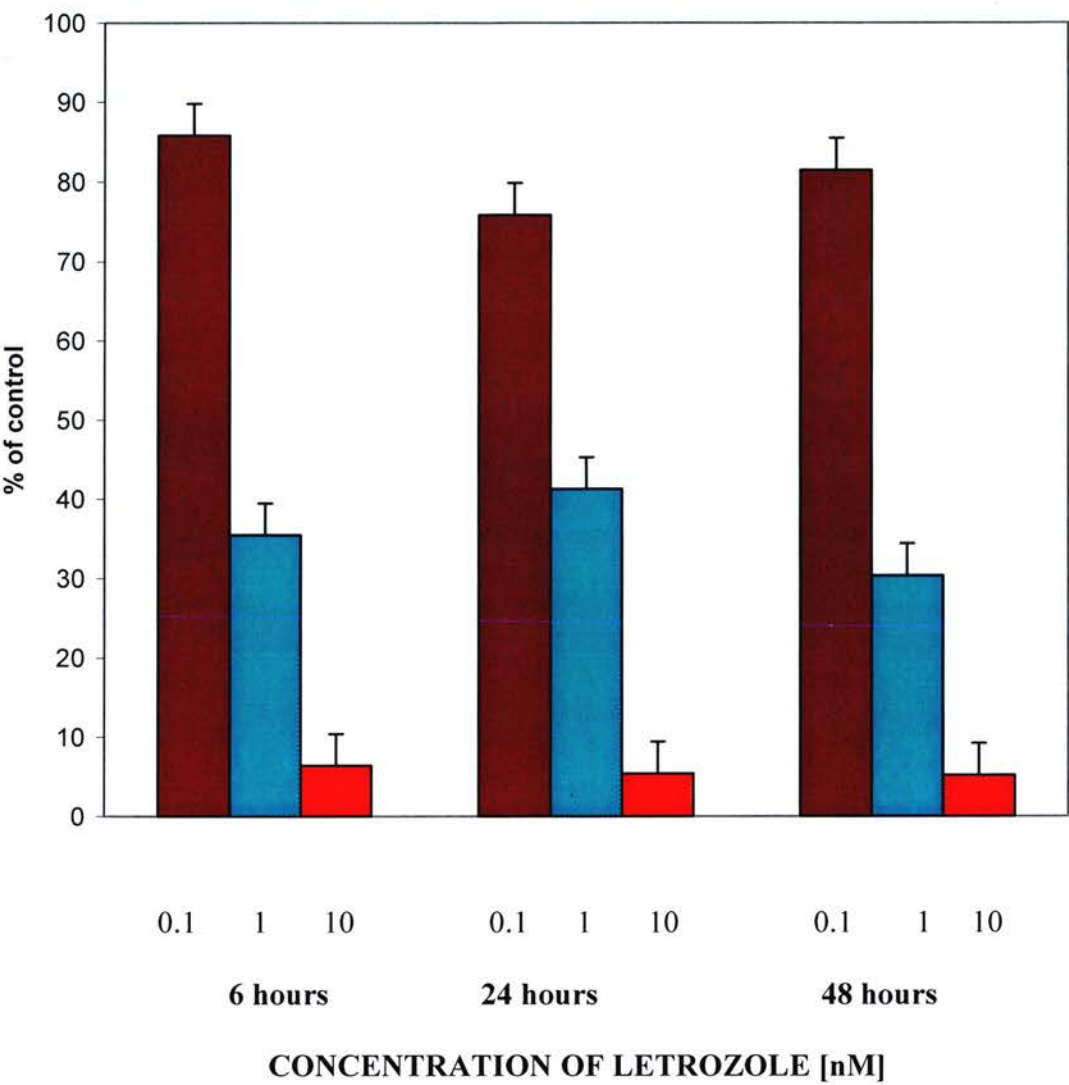
Cells were preincubated in the presence of dexamethasone and aromatase activity was determined at 6, 24 and 48 hours after addition of aromatase inhibitors to the culture media and activity was determined in the presence of aromatase inhibitors – letrozole and exemestane. The results are shown in **fig 12a and 12b**.

Letrozole produced inhibition of aromatase activity at all concentrations studied. The maximum inhibitory effect was seen at 1nm. Letrozole continued to exert inhibitory effect when incubated between 6 to 48 hours. It is observed that letrozole produced a similar response as seen previously irrespective the duration of incubation.

Exemestane also produced inhibition of aromatase activity at all concentrations studied. The maximum inhibitory effect was seen at 10nm. Exemestane when incubated between 6 to 48 hours produced inhibition of aromatase activity. It was also noted that exemestane produced a consistent inhibitory effect irrespective of the duration of incubation.

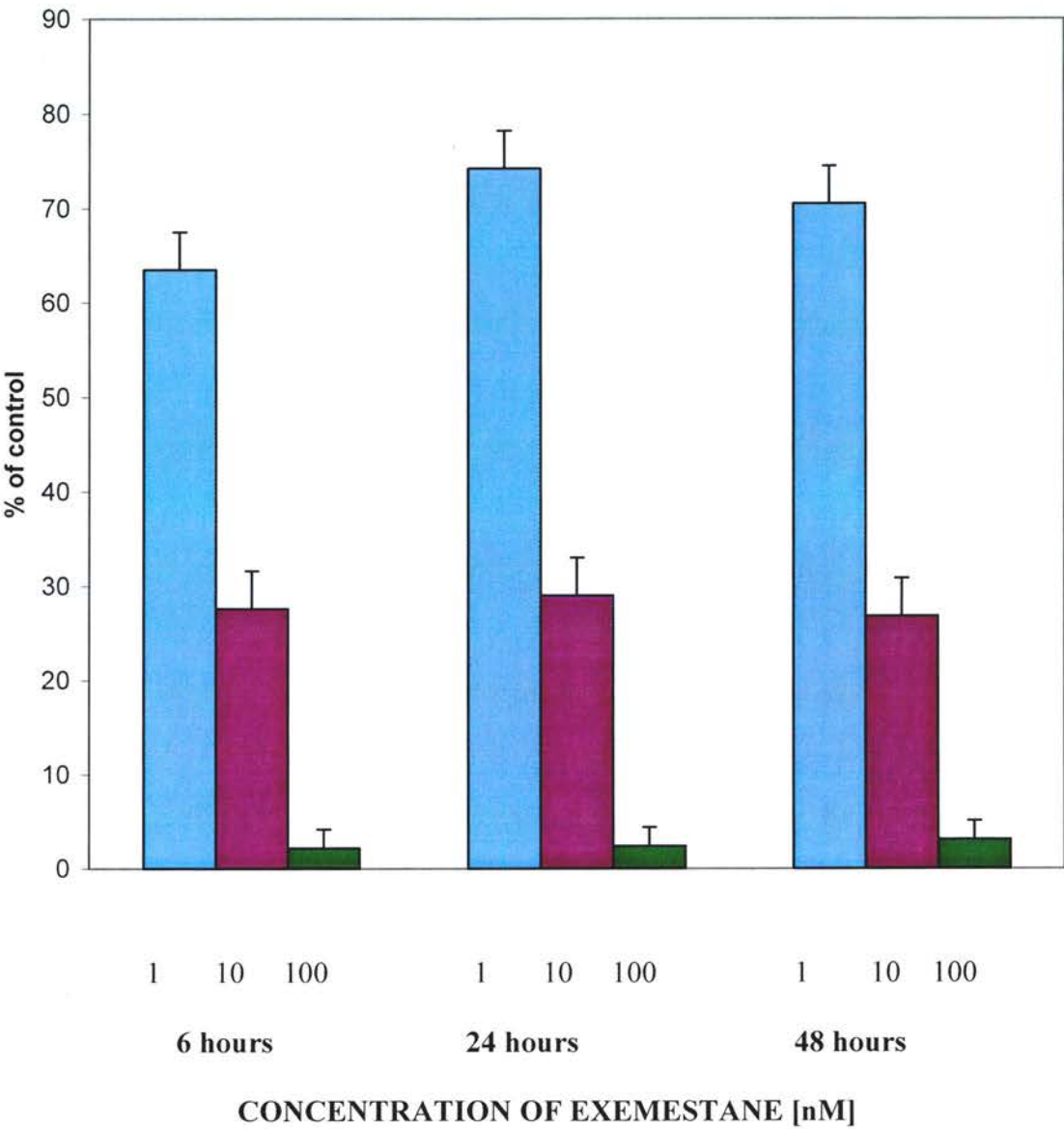
Thus the consistent inhibitory effect observed in the presence of both letrozole and exemestane might have important implication when used in treatment of breast cancer, which shall be discussed later.

**Fig 12a: Time course of fibroblast cells treated with letrozole**





**Fig 12b: Time course of fibroblast cells treated with exemestane**



## DISCUSSION

The results from incubation of culture of breast adipose tissue fibroblasts confirm the remarkable efficacy of the newly developed aromatase inhibitors. This is most readily seen with aromatase activity being effectively inhibited in the invitro study.

Both steroidal and nonsteroidal aromatase inhibitors inhibited aromatase activity in dose-related fashion, with concentrations of aminoglutethimide required at micro molar range, while others including anastrozole, letrozole, exemestane and formestane were able to inhibit at nanomolar concentrations. It confirms the potency of the newer generation of aromatase inhibitors with the newer inhibitors at least 100-fold more potent than aminoglutethimide.

Dexamethasone and cyclic AMP were used to induce aromatase activity and the reasons for using, these inducers will be discussed.

Isolated stromal cells from breast cancer tissue contains aromatase enzyme, which can be stimulated to nearly four orders of magnitude by dexamethasone, phorbol esters and cyclic AMP [Simpson ER, 1983]. Dexamethasone was the most effective steroid in stimulating aromatase activity and this effect was blocked by actinomycin or cyclohexamide [Simpson ER, 1983]. In this study, preincubation of fibroblast cultures with various aromatase inhibitors in the absence of dexamethasone illustrated a five fold increase in basal levels of aromatase (which were low). The stimulatory effect of dexamethasone was apparent after a preincubation period of 4 hours and was maximal after 24 hours with no further increase in activity thereafter [Simpson ER, 1981]. In this study preincubation was carried out for 18 hours as this duration has been previously

confirmed to produce optimal stimulatory effect [Miller WR, 1993]. Inclusion of dexamethasone for 18 hours in the culture media markedly induced aromatase activity with the effects being dose related between concentrations  $10^{-7}$  and  $10^{-8}$ . The stimulatory effects of dexamethasone were noted only in the presence of foetal calf serum (FCS) in the culture medium [Simpson ER, 1983]. Activation of aromatase activity by foetal calf serum in the presence of glucocorticoids is not only confined to adipose tissue but also has been reported with skin fibroblasts and hepatocytes [Lanoux MJ]. Various reasons have been postulated to be responsible for the inductive effect of glucocorticoids. Glucocorticoids may increase aromatase activity by binding to specific receptor, which induces synthesis of specific proteins the cytochrome P450. It is also known to activate sequences and bind transcription factors of the STAT family. This may induce transcription of mRNA, which results in synthesis of new protein cytochrome P450.

In this study cyclic AMP was also used to potentiate aromatase activity. Fibroblast cell cultures were preincubated with cyclic AMP for 72 hours and in the absence of foetal calf serum. It has been previously demonstrated that cyclic AMP stimulated aromatase activity after 48 hours and the presence of foetal calf serum, inhibited this stimulatory effect by 70% [Mendelson CR 1986]. Hence all the experiments were conducted in the absence of foetal calf serum. The inhibitory effect of foetal calf serum may be due to the presence of certain factors that inhibits aromatase activity by acting on one or more complex components of the enzyme complex that are increased by cyclic AMP. The fact that serum inhibited the stimulatory effect of aromatase activity by cyclic AMP and the time course of induction were markedly different for dexamethasone and cyclic AMP

and are suggestive of different molecular events mediating aromatase induction with both these agents [Simpson ER, 1983].

The strength of the fibroblast cell culture model used in this study, is that it simulated both invitro and invivo conditions. Cultures of mammary fibroblasts were preincubated with aromatase inhibitors (to simulate patient treatment) and then assayed in the absence of drugs as were the breast tissues from treated patients (mimic exvivo studies). Additional cultures were set up using the design of incubating the cultures in the absence of aromatase inhibitors but assaying for aromatase activity in the presence of aromatase inhibitors (mimics the invivo studies). The study model adopted has the advantage that invivo conditions could be simulated using the invitro setting.

Interestingly, all the drugs, both type I and type II inhibitors – exemestane, formestane and letrozole, anastrozole, fadrazole and aminoglutethimide demonstrated significant inhibitory effects on incubation ( $p < 0.001$ ). Letrozole was more potent than other type II inhibitors ( $p < 0.001$ ) and exemestane was more potent than formestane ( $p = 0.039$ ). This inhibitory effect demonstrates the potency of the newer generation of aromatase inhibitors. The inhibitory effect on aromatase activity was greater with  $100\mu\text{m}$  of aminoglutethimide,  $10\text{nm}$  of letrozole while other inhibitors - anastrozole, fadrazole, exemestane and formestane produced greater inhibitory effect at  $100\text{nm}$  concentration. This demonstrates that small doses may be required for treatment (invivo) with both type I and II inhibitors, except with aminoglutethimide which may require larger doses. Aminoglutethimide also lacks the specificity which may be due to its interference with other cytochrome P450 systems [Santen RJ, 1981]. The newer generation drugs such as letrozole and anastrozole have relatively minimal effects on other cytochrome P450

containing enzymes including those involved in glucorticoids synthesis [Trunet PF; Masamura S, 1995]. This would mean no corticoid replacement is required when using the newer generation of aromatase inhibitors.

In the preincubation setting letrozole produced a bell shaped dose response curve with inductive effect at lower concentrations of 2-20nm ( $p<0.005$ ) and inhibition at higher doses 100-1000nm ( $p<0.0005$ ). This response pattern was similar to that observed by Miller et al [1999]. In contrast when assays were performed in the presence of letrozole consistent inhibitory effect was seen. This experimental setting is similar to ex-vivo setting and may have important clinical implications which will be addressed later. Interestingly the inhibitory effect seen with letrozole at 100nm is less than that seen with the same concentration when present in the assay phase.

In comparison to letrozole, anastrozole on preincubation produced persistent enhanced activity at all concentrations studied ( $p<0.0005$ ). It reached a plateau effect with 1nm and this remained persistent at 100nm. However when assays were performed in the presence of anastrozole consistent inhibitory effect was produced and the inhibitory potential was less than that of letrozole ( $p<0.001$ ). Fadrozole on preincubation displayed consistent enhancement of aromatase activity similar to other drugs in the same group ( $p=0.008$ ). The effects produced by fadrozole could not be compared to other studies as no similar experiments were conducted in breast fibroblasts. However Harada et al [1998b] observed similar paradoxical effects in JEG3 choriocarcinoma cell culture lines. Such effects may reflect the selective affinity of type II agents for the cytochrome P450 in aromatase. Aminoglutethimide also produced inductive effect in the preincubative setting ( $p=0.058$ ) and inhibitory effect on incubation but requiring micro molar

concentrations. This is similar to the response observed previously [Miller WR, 1987]. This may have clinical implication in the dosage of drugs used as small dosage of new aromatase inhibitors would be sufficient to achieve oestrogen suppression while large doses of aminoglutethimide may be needed to be obtain the same suppressive effect. Enhanced aromatase activity following exposure to aminoglutethimide has also been reported in other culture systems [Miller WR, 2000].

Interestingly when type I drugs were studied, both exemestane and formestane ( $p < 0.0005$ ) produced consistent inhibitory effect at all concentrations studied on preincubation. The type I agents produced lesser inhibitory effect in the inductive phase than when the drug was measured in the assay itself. The persistent inhibitory effect seen with type I drugs may reflect the irreversible binding of the drug, which may be totally independent of the amount of aromatase produced [Brodie AMH, 1986].

Letrozole was more potent than other type II inhibitors with 10nm producing greater inhibitory effect ( $p < 0.001$ ). This has clearly demonstrated the superior potency of letrozole than the other type II drugs. Dixon et al have observed similar potent effects of letrozole in the clinical setting. They also observed that letrozole alone was able to retain some of its inhibitory potential at certain concentrations. This may have important implication in the clinical sequence of the use of aromatase inhibitors. Probably potent drugs such as letrozole (at certain concentrations) must be commenced initially and with development of drug resistance type I drugs could be used.

When preincubated with type II inhibitors, a paradoxical increase in aromatase activity was observed and when incubated in the presence of inhibitors (either type I or type II),

all the inhibitors markedly and consistently inhibited aromatase activity. This is contradictory to the normal inhibitory potential of aromatase inhibitors both in the clinical and invivo situations. Similar paradoxical results have also been reported in culture of JEG3 and HepG2 cells in which type II inhibitors such as fadrozole and vorozole enhanced aromatase activity [Harada N, 1998b]. Thus the persistent paradoxical effect produced in different cell culture models may have important therapeutic implications.

Co-agents like glucocorticoids are well known to induce aromatase activity. Therefore it was necessary to prove that the paradoxical inductive effect of aromatase inhibitors was not a feature of the inducers. Primary culture studies were preincubated with dibutyryl cyclic AMP to induce aromatase activity and experiments were conducted in similar settings. The results of this study are not comparable as no similar experiments have been conducted to date.

Incubation which simulated patient treatment produced marked inhibition of aromatase activity with type I inhibitors, while type II inhibitors produced a paradoxical increase in aromatase activity. When assayed in the presence of inhibitors, both steroidal and nonsteroidal aromatase inhibitors, markedly inhibited aromatase activity. The aromatase inhibitor anastrozole produced greater inductive effect when compared with letrozole at all concentrations studied ( $p < 0.0005$ ). Letrozole alone demonstrated inhibitory effect at higher concentrations in comparison to other drugs in the same group ( $p = 0.041$ ). This may illustrate the superior potency of letrozole which alone is able to retain its inhibitory effect at certain concentrations.



Interestingly, on preincubation and incubation with type I inhibitors aromatase activity was inhibited at all concentrations studied (1-1000nm). Exemestane produced greater inhibitory effect in comparison with formestane ( $p < 0.05$ ) and appears to be more potent than formestane and this may be ascertained from the results of the new clinical trials.

The paradoxical inductive effect on aromatase activity observed with type II drugs on preincubation was demonstrated both with dexamethasone and dibutyryl cyclic AMP. This has clearly demonstrated that paradoxical inductive effect observed in certain circumstances with type II agents is not a feature of the inducers. Though the two inducers dexamethasone and cyclic AMP mediate induction of aromatase activity by different molecular events, the effects produced by the steroidal and non steroidal aromatase inhibitors is consistent proving that the effects displayed by them is not influenced by the inducers used.

The inductive effects of aromatase inhibitors observed under certain conditions in cell culture model may have important clinical implications in the use of these drugs. Thus the type II drugs may be associated with hormonal escape phenomenon with the long term use and the type I inhibitors may be indicated in such situations.

Various reasons for the paradoxical effect exhibited by type II drugs can be postulated. It can be a reflection of the different mechanism of action of the two groups of drugs or it can be due to alteration at a molecular level. The lack of full inhibition and the enhanced activity of type II inhibitors may reflect the reversible nature of these inhibitors and its ability to induce aromatase mRNA/stabilize aromatase protein.

The ability to induce aromatase mRNA/stabilisation of aromatase protein has been reported by Harada et al [1998b] in JEG-3 cells (invitro) and in mice (invivo) and by



Chen et al [1999]. These effects have been attributed to stabilisation of the enzyme protein by protection from proteolytic digestion. Additionally, such inhibitors might stabilise the aromatase enzyme by preventing the destructive peroxidation associated with hydroxylation.

The substrate androstenedione is known to protect the steroidal inhibitors from inactivation and it would be of importance to determine its effect invitro. The steroidal compounds are analogues of the substrate androstenedione and compete with the substrate for the enzyme-binding site. In addition they may be suicide inhibitors, which bind covalently to the enzyme-binding site in an energy dependent manner. This mechanism has been demonstrated invitro by increasing the duration of preincubation with the inhibitor prior to measurement of aromatase activity [Brodie AMH, 1981]. Fibroblast cultures were preincubated with the substrate (androstenedione) in the presence of aromatase inhibitors (letrozole and formestane) and assayed in the absence of inhibitors. The physiological concentration of androstenedione had no influence on the inhibition. Addition of substrate did not influence the paradoxical stimulatory effect observed with letrozole and the inhibitory effect of formestane (invitro). Harada et al [1999] believed that aromatase inhibitors and substrates bind competitively to same binding sites within the aromatase molecule to form conformationally tight complexes, which would be resistant to proteolytic degradation. The pharmacodynamics of steroidal inhibitor is determined partly by the continued presence of the inhibitor and partly by the synthesis of new enzyme. Thus the use of this type of inhibitor may result in substantial intracellular build up of the drug, which would need to be addressed in the clinical setting.

The influence of variation in time on the effects of aromatase inhibitors was also studied. The fibroblasts were treated with letrozole and exemestane (1-1000nm) over 6 - 48 hours and assayed in its absence. Both consistently inhibited aromatase activity with time having no influence on its effects. However Harada et al [1998b] observed a time dependent increase in aromatase protein using JEG cells with the non steroidal agents – fadrozole, vorozole and pentrozole and mRNA levels in the cells remain unchanged during the inhibitor treatment. It was therefore suggested that aromatase inhibitors increased aromatase protein through stabilisation and reduced protein turnover.

Thus the inductive effects of aromatase inhibitors observed under certain conditions (invitro setting) may have important clinical implication. Long term use of type II aromatase inhibitors may be associated with hormonal escape phenomenon which may necessitate the use type I inhibitors in such situation.

## 6. Exvivo studies

### 6.1 Treatment with letrozole

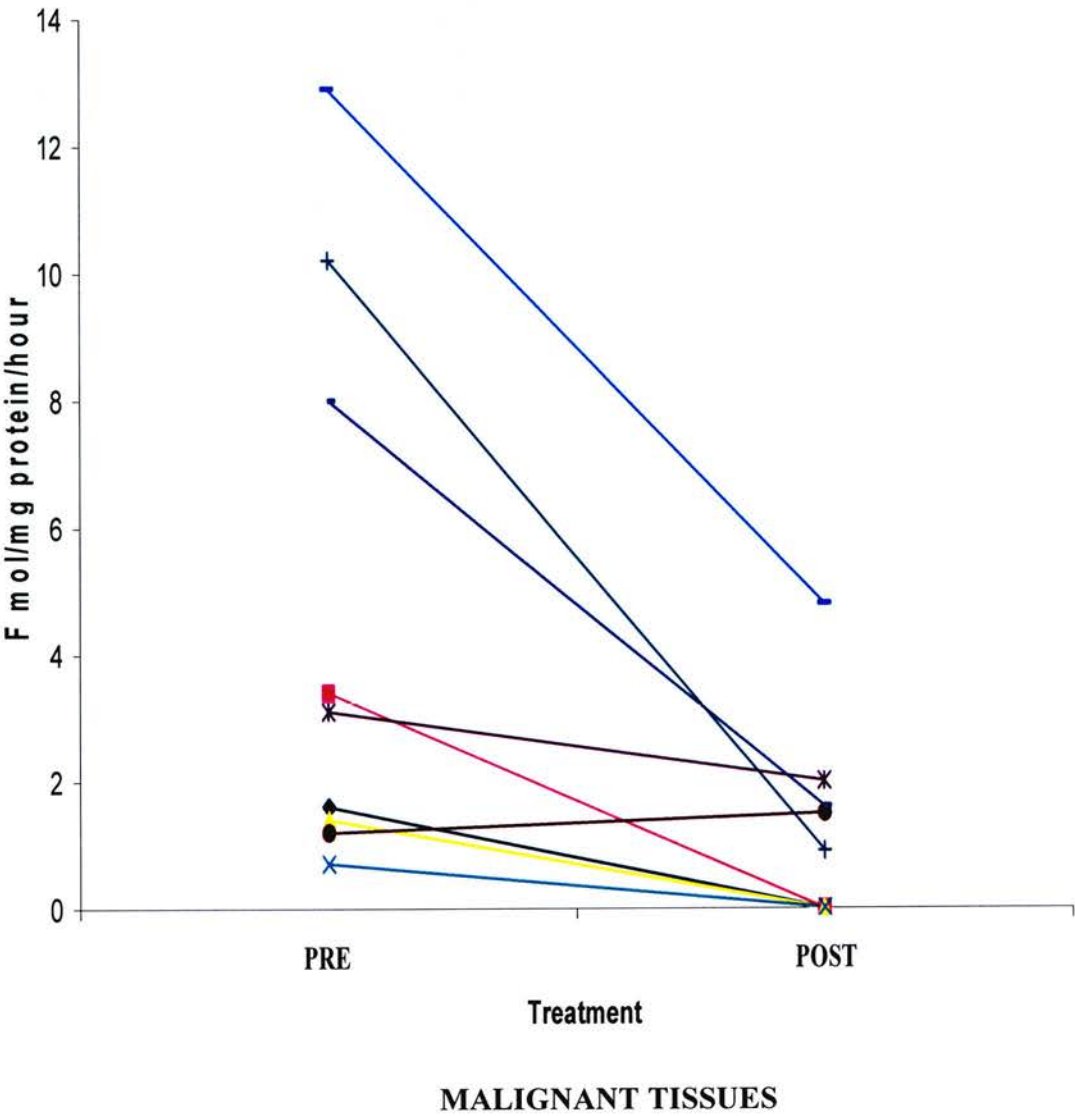
Aromatase activity was measured (invitro) in nine paired samples of malignant and non-malignant tissues obtained before and after treatment with letrozole 2.5mg/day for 3 months. Particulate fractions were prepared and aromatase activity was determined as described previously. The results are summarised in **figure 13 and 14**.

All patients had measurable aromatase activity in pretreated tumour samples varying between 0.5 to 3.38 fmol/mg protein/hour. Eight patients had decreased aromatase activity and only one patient demonstrated paradoxical increase in activity following treatment with letrozole. The aromatase activity in responding tumours was almost negligible following treatment. The changes following treatment with letrozole was statistically significant ( $p=0.017$ ) with a mean difference of 3.52 between pre and post treated tumour samples. This demonstrates the potency of letrozole in reducing aromatase activity in tumour tissues. Only one tumour demonstrated paradoxical induction of aromatase activity. Aromatase activity increased from 1.2 fmol/mg protein/hour in pretreated sample to 1.5 fmol/mg protein/hour in post-treated tissue and this change was not statistically significant ( $p=0.46$ ). One patient had paradoxical increase in aromatase activity in tumour tissue also demonstrated similar effect in the nonmalignant tissue following treatment although the aromatase activity in the nonmalignant tissue was higher than the tumour itself.

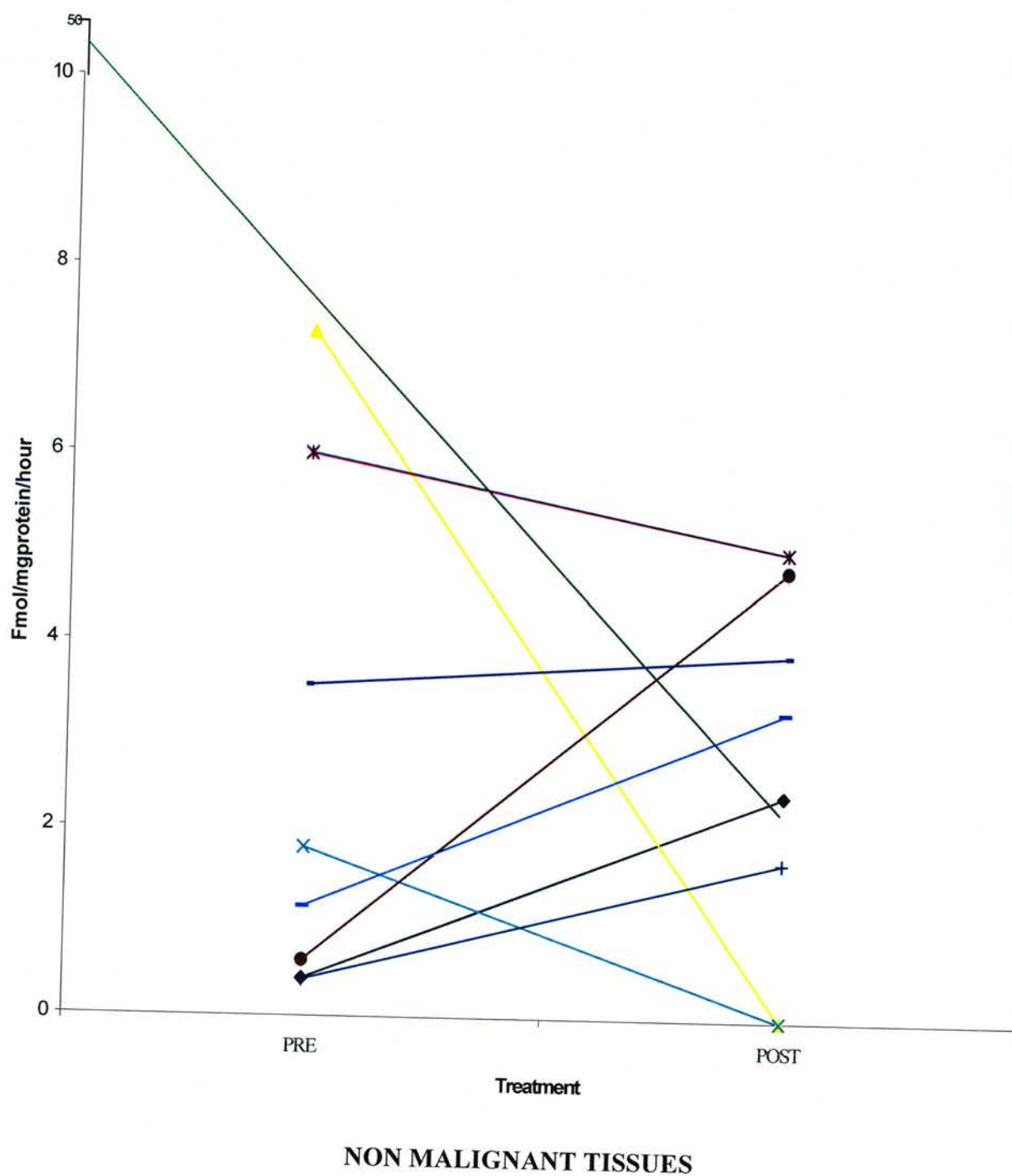
In nonmalignant breast tissue measurable aromatase activity was demonstrated in all the pretreated tissue samples. Nonmalignant tissues had a variable amount of aromatase

activity ranging between 0.3 - 47.3 fmol/mgprotein/hour. It was interesting to note that one sample of non malignant tissue had initially very high measurable aromatase activity (47.3 fmol/mgprotein/hour) which decreased following treatment. Significant decrease in aromatase activity was observed in five patients and paradoxical increase in activity was observed in four patients following treatment. The changes following treatment with letrozole in non malignant tissues was statistically insignificant ( $p=0.36$ ) with a mean difference of 4.15 between pre and post treated tumour samples. The paradoxical increase in activity was mostly observed in specimens, which had initially low aromatase activity. The paradoxical increase in aromatase activity was however statistically insignificant ( $p=1$ ). Thus the non malignant tissues have demonstrated variable response following treatment with letrozole.

**Fig 13: Aromatase activity in malignant breast tissues before and after treatment with letrozole**



**Fig 14: Aromatase activity in nonmalignant breast tissues before and after treatment with letrozole**



## 6.2 Treatment with anastrozole

Aromatase activity was measured in malignant and nonmalignant tissues (invitro) in patients before and after treatment with anastrozole (1mg/day) for 3 months. Particulate fraction of tumours obtained from two patients and nonmalignant tissues from five patients were prepared and aromatase activity was determined as described before. The results are shown in **Table 15 and Fig 15**.

Both the tumour samples had significant aromatase activity in the pretreated tissues and higher aromatase activity was expressed in comparison to nonmalignant tissues. Following treatment both the tumours studied, displayed a significant decrease in aromatase activity ( $p < 0.05$ ).

The nonmalignant tissues had demonstrated significant aromatase activity in pretreated tissue samples in all but one, which had almost no measurable activity. Four had decrease in aromatase activity following treatment ( $p = 0.01$ ). The mean difference between pre and post treatment sample was 2.50. Paradoxical increase in activity was observed following treatment in one sample ( $p < 1$ ). The nonmalignant tissues that demonstrated a paradoxical induction in aromatase activity had initially no measurable activity.

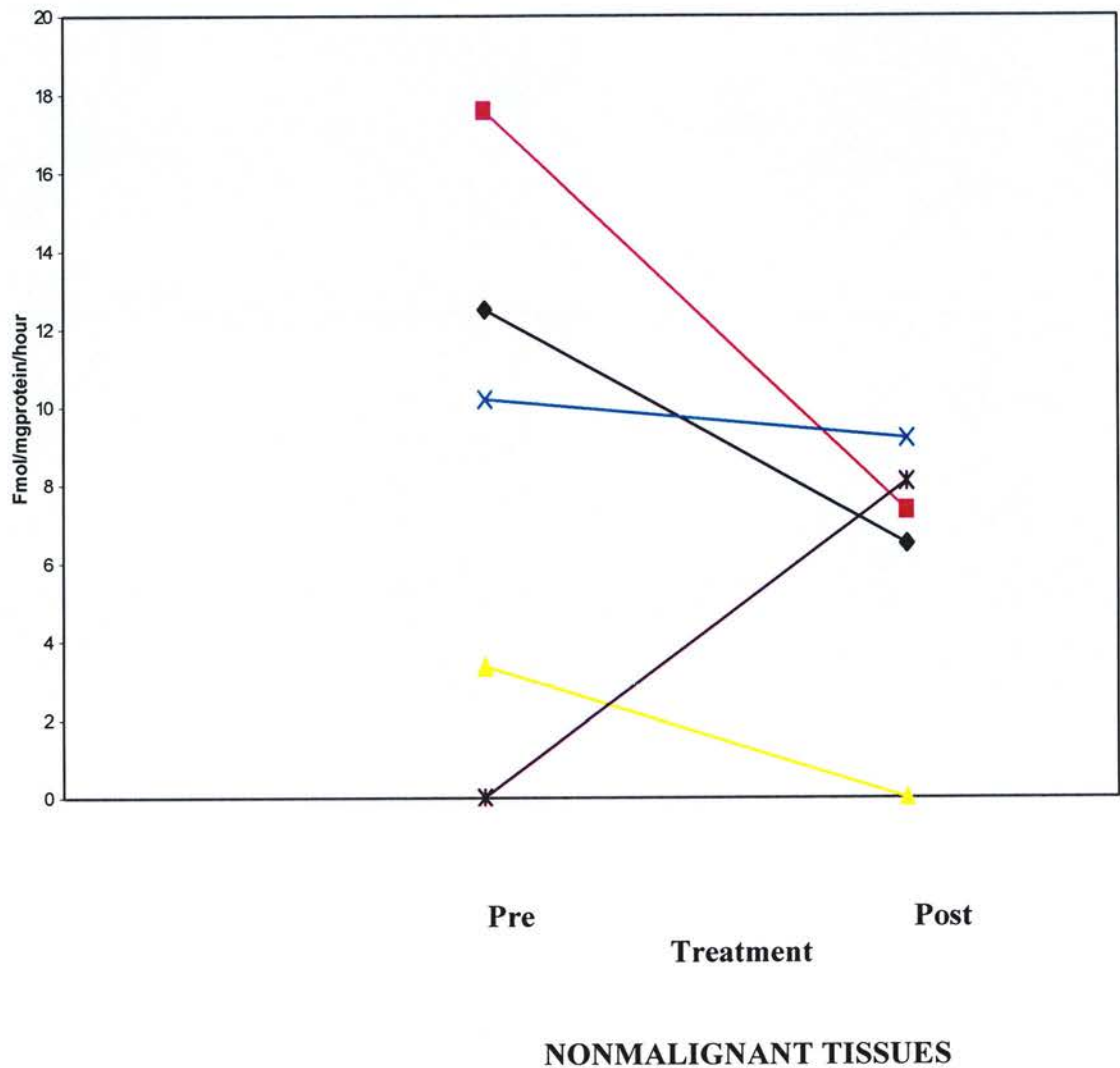
Thus the malignant and nonmalignant tissues studied displayed the expected inhibition in aromatase activity following treatment though the numbers studied were small.

**Table -15: Aromatase activity invitro in paired samples of breast cancer before and after 3 months of treatment with anastrozole**

PATIENT	PRE/POST	ACTIVITY
	Treatment	fmol/mg protein/hr
J	Pre	52.1
	Post	5.5
K	Pre	17.6
	Post	6.3



**Fig 15: Effects of 3 months of treatment with anastrozole on aromatase activity in nonmalignant tissues**



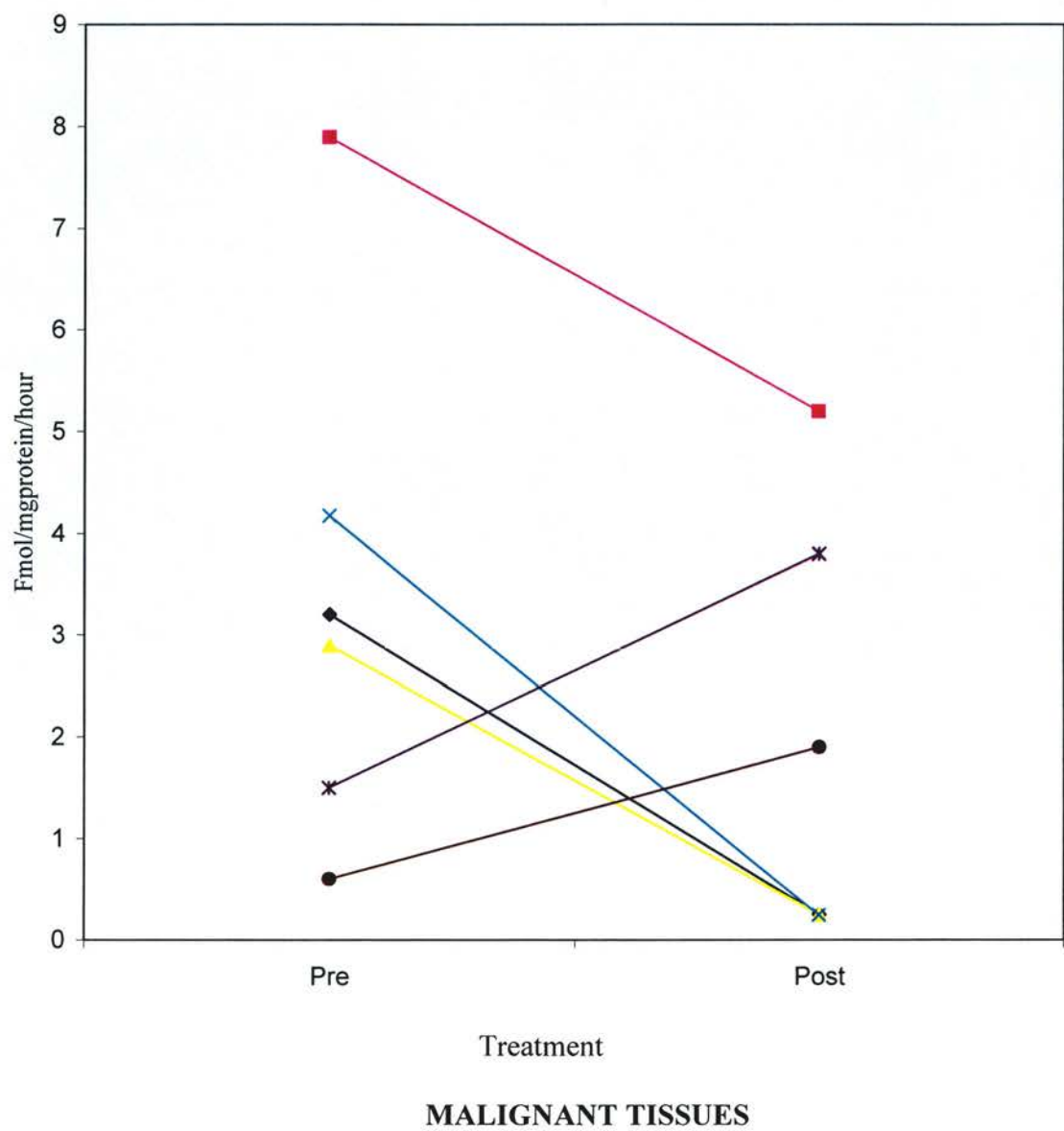
## 6.1 Treatment with exemestane

Six postmenopausal women were treated with exemestane (25 mg) for 3 months. Particulate fractions of malignant and non malignant tissues were prepared. Aromatase activity was determined. The results are shown in **fig 16**.

Aromatase activity varied between 0.6 - 7.9 fmol/mg protein/hour in the pretreated tissues samples. Similar to other aromatase inhibitors pretreated tumours had measurable aromatase activity. Four patients had a decrease in activity and two patients had a paradoxical increase in activity following treatment. The changes following treatment with exemestane in malignant tissues was significant ( $p < .05$ ). The mean difference between pre and post treated tumour samples was 1.44. The two samples which demonstrated paradoxical increase in aromatase activity following treatment had also initially low aromatase activity. The paradoxical increase in aromatase activity in malignant tissue samples was statistically insignificant ( $P < 1$ ).

Thus most malignant tissues treated with exemestane demonstrated a decrease in aromatase activity following treatment.

**Fig 16: Effects of 3 months of treatment with exemestane on aromatase activity in malignant tissues**



## **Treatment with aromatase inhibitors**

Aromatase activity was well demonstrable in all the tumour samples studied before treatment. Following treatment decrease in aromatase activity was seen in most of them. This could be consistent with the observation that tumour tissues have high aromatase activity and significant suppression occurs with the use of third generation aromatase inhibitors. The changes following treatment letrozole, anastrozole and exemestane were statistically significant between pre and post treated tumour samples ( $p=0.017$ ,  $p=0.01$ ,  $p<0.05$ ). This demonstrates the potency of the third generation aromatase inhibitors in reducing aromatase activity in tumour tissues. The decrease in aromatase activity following treatment occurred in majority of the tumours samples treated; one of nine treated with letrozole, two of two treated with anastrozole, four out six treated with exemestane. It is interesting to note the mean difference between pre and post treated tumour samples treated with letrozole was greater (3.52) in comparison with exemestane (1.44).

Paradoxical increase in activity could only be seen with one letrozole and two exemestane treated tumours (statistically insignificant). The paradoxical increase in aromatase activity observed in one letrozole treated tumour had produced a similar response in the nonmalignant tissue also. The paradoxical increase in aromatase activity was seen in tumour tissues, which had initially low aromatase activity before treatment. In contrast, both the anastrozole treated tumours showed the expected inhibition of aromatase activity following treatment.

Nonmalignant breast tissues demonstrated variable aromatase activity. Paradoxical increase in activity was observed in four patients following treatment with letrozole and one treated with anastrozole. The changes following treatment in non malignant tissues was statistically significant with anastrozole ( $p=0.01$ ) and insignificant with letrozole ( $p=0.36$ ). On the contrary, most of the nonmalignant tissues demonstrating paradoxical increase in aromatase activity had initially low activity in comparison to its tumour counterparts. Decrease in aromatase activity was observed in five patients treated with letrozole ( $p=1$ ) and four patients treated with anastrozole ( $p<1$ ), although was not statistically significant. The paradoxical increase in activity was mostly observed in specimens, which had initially low aromatase activity. Thus the non malignant tissues have demonstrated variable response following treatment.

The reason for paradoxical increase in aromatase activity may be that, invitro estimates of treated specimens may under estimate the decrease of inhibition produced invivo: this makes the use of protocols that accurately measure of in situ activity essential when assessing the putative efficacy of individual inhibitors. Although observation in experimental test systems is informative, they may not necessarily reflect events occurring within the body. Other possible causes includes that invitro assays may underestimate the potency of type II inhibitors. Even though aromatase activity may be blocked by the drugs in situ, the reversible nature of the association of type II drugs may mean that aromatase activity is released from its inhibition. This would not be the case for irreversible type I inhibitors which have also demonstrated failure of total inhibition of aromatase activity in certain tumours. Thus failure of suppression of aromatase activity has been demonstrated in certain malignant and nonmalignant tissues following

primary treatment with letrozole, anastrozole and exemestane. The reasons for such paradoxical mechanism are discussed in detail in the following chapter.

## DISCUSSION

Postmenopausal patients with large operable oestrogen receptor (ER) positive primary breast cancers were treated with aromatase inhibitors - letrozole, anastrozole and exemestane. Experiments were undertaken using paired specimens of both malignant and nonmalignant breast tissues obtained before and after treatment. The effects of aromatase inhibitors were assessed, by comparing aromatase activity before and following treatment.

Although different methods can be used to measure aromatase activity, biochemical assay was used in this study. The bias in variability in the amount of tissue used was eliminated by measuring the protein content and results were expressed as fmol/mg protein/hour. The study sample consisted of a group of 16 postmenopausal patients. Aromatase activity varied in pre and post treated malignant and nonmalignant tissues studied. Possible explanations for the variation will be addressed.

Aromatase activity was demonstrated in all the tumour tissues studied. This finding was similar to the observation made in other studies with measurable aromatase activity both in normal and malignant breast tissues [Perel E, 1982; Reed MJ, Lu Q]. Aromatase activity detected (0.3 – 3.72 pmol /g protein /hour) in pretreated tumour tissues was similar to the observation made by Lipton et al. In this study higher aromatase activity was demonstrated in malignant tissues while the activity was variable in nonmalignant tissues. This finding was similar to that of James et al, who demonstrated higher aromatase activity in breast tumours (invitro). Previous studies have shown a significant

correlation between aromatase activity and tumour bearing quadrants [Miller WR, 1987] but this was not addressed as this was beyond the scope of this thesis.

Results of this study showed marked decrease in aromatase activity following treatment in most of the tumour tissues while variable response were observed in nonmalignant tissues. The majority of tumours treated with aromatase inhibitors have showed a consistent inhibitory effect although paradoxical increase in activity was observed in three patients. Marked decrease in activity following treatment was observed both with letrozole ( $p=0.017$ ) and anastrozole ( $p<0.05$ ) in all but in one patient. This correlates with the findings of Dixon et al where marked suppression of aromatase activity was noted in letrozole treated patients (invivo). The exemestane treated patients also demonstrated a decrease in activity following treatment except for two patients ( $p<.05$ ). Thus the third generation aromatase inhibitors have demonstrated their potency in reducing aromatase activity following treatment in most of the tumour samples studied.

In nonmalignant breast tissues the degree of decrease with treatment was less marked and paradoxical increases in activity were seen in cases in which aromatase activity was initially low. Although there was a paradoxical increase in aromatase activity following treatment it was noticed to be statistically insignificant, although the numbers treated were small. Interestingly all these patients had a clinical response, which may reflect the low sensitivity and specificity of the invitro assays itself.

Possible explanations for the variation in aromatase activity before and after treatment are addressed.

A comparison was made assessing aromatase activity before and after treatment in a retrospective study of patients demonstrating a significant reduction of aromatase in



responding tumours and a significant increase in non-responding tumours. These changes were observed when the clinical behaviour of the tumours was already known. Interestingly all the tumours had a clinical response but for one. This may reflect the fact that invitro estimation may not be a direct reflection of invivo activity. The timing of obtaining specimen may be important as these were all taken at the end of 3 months, which was an arbitrary period of treatment. It must also be noted that the study was conducted in specimens stored in liquid nitrogen.

Although aromatase inhibitors are used to treat breast cancer, treatment has found to increase the level of aromatase in the breast tissue of some patients as observed in this study. Changes in aromatase activity following treatment have been previously studied invitro to other aromatase inhibitors mainly aminoglutethimide. Paradoxical increase in aromatase activity in tumours from patients treated with aminoglutethimide has been initially observed by Miller et al [1987]; this activity was still shown to be sensitive to aminoglutethimide, by demonstrating inhibition after invitro incubation with the drug.

The different types of assay, the problems in measurement of aromatase activity are addressed.

Bezwoda et al have demonstrated that tumour aromatisation in breast cancer tissue was a useful measurement in predicting response to therapy [1987]. In this study aromatase activity during neoadjuvant therapy has been useful in predicting response to treatment in malignant tissues while variable effect has been observed in nonmalignant tissues.

Aromatase activity during neoadjuvant therapy would be a useful predictor of response to treatment if reliable method of assessment were available. The present accepted 'gold standard' method of estimation of aromatase activity is biochemical assay, which may

not reflect the insitu activity [Shenton KC]. The vitro assays performed require fresh or frozen tissue and are more cumbersome. They may also not accurately reflect the invivo activity. It must be noted that parallel studies using product isolation assay were not performed in this study. It may be possible that some tumours may release  $^3\text{H}$  from 1beta androstenedione into aqueous phase without being able to detect radioactively labelled oestrogen by product formation. However the use of product isolation assay may also display the phenotype with low / no aromatase activity despite the amount of mRNA transcripts.

Assessment of invivo activity has been carried by Reed and Dixon et al. It requires administration of radioactive material (invivo) and assessment is carried out in the presence of the administered drug [Santen RJ, 1982]. The invivo technique is invasive, involves use of radioactively labeled infusions and an extra procedure in patient management and could also be influenced by paracrine and local factors. Immunohistochemistry techniques can be performed in paraffin embedded material. It requires reliable antibodies and reproducible scoring systems. Immunohistochemical assays are controversial as the exact localisation of aromatase activity is yet to be determined. Attempts have been made to develop reliable immunohistochemistry techniques and a multicentre collaborative group has been established to generate and validate new aromatase monoclonal antibodies [Sasano H, 2003]. Reverse transcriptase polymerase chain reaction can demonstrate the presence of aromatase RNA but it requires amplification, quantitation and has cumbersome handling techniques.

Another indirect method of assessment is by measuring circulating serum oestradiol. All of the current third-generation aromatase inhibitors (exemestane, anastrozole, letrozole)

reduce the amount of circulating serum oestradiol to such a low level, that it can barely be measured by commonly used assays. Furthermore, it is unclear whether the differences in the serum levels are important in predicting or controlling response to antiaromatase agents in breast cancer patients. However, serum oestrogen levels were not measured as it was beyond the aim of this thesis. As oestrogen levels in the circulation do not necessarily reflect those in the breast and because local aromatase activity may be affected by antiaromatase agents, it is essential to determine the effects of aromatase inhibitors on the endocrinology within the breast.

Aromatisation is considered to play an important role in development of breast carcinoma and hence methods to detect aromatase must be highly sensitive and specific. The role of intratumoural aromatase is becoming more important as the use of aromatase inhibitors continue to increase in clinical setting [Brueggemeier RW] and it would be a predictive marker to determine the response to treatment. Therefore in future, development of a reliable technique for the assessment of intratumoural aromatase is highly desirable in order to optimise the treatment modalities.

The reasons for the variable result seen in this study with malignant and nonmalignant tissues may be related to methodology. Invitro studies yield results, in which inhibition is incomplete or activity may be increased, other endocrinological data on circulating oestrogens or whole-body perfusion studies suggest that inhibition of peripheral aromatase is almost total [Geisler J, 1998]. Invivo assays have demonstrated a decrease in activity following treatment with aromatase inhibitors [Miller WR, 2001]. Thus invitro assays performed from inhibitor treated patients may not accurately reflect the degree of inhibition produced invivo. Exvivo studies of tissue aromatase indicated that

such measurements consistently underestimate the inhibitory potential of reversible non-steroidal agents. Although aromatase activity may be blocked by non-steroidal agents *in situ*, *invitro* assays are performed without the addition of inhibitors so aromatase activity can be released from its inhibition due to the reversible nature of type II drugs (but not type I). It could also be due to the fact that tumour aromatase activity may be influenced by systemic and local factors. *Invitro* measurement of tumour aromatase activity may probably not reflect activity *invivo* due to potentiating effects of growth factors, cytokines and proteins.

The various mechanisms, which may produce paradoxical response following treatment, are addressed.

Paradoxically breast cancers from patients treated with aminoglutethimide display elevated levels of aromatase; comparisons of sequential tumour samples taken before and following treatment show that aromatase may be induced as much as 25 fold [Miller WR, 1988]. Addition of the same drug to cultures of fibroblast cell lines also produced a profound increase in aromatase activity. These effects would be consistent with the drugs ability to induce cytochrome P450 hydroxylase [Santen RJ, 1981]. Thus elevated aromatase could potentially reduce the drug efficacy and under these circumstances individuals might be beneficially treated with type I aromatase inhibitor.

The increase in aromatase activity in non-responding tumours may be a reflection of the growth of unresponsive tumour. There is also a possible concern that aromatase inhibitors can lead to stimulation of tumour growth in this subgroup of patients. It may be that the cells may represent resistant clones, which were more in non-responding tumours although the number in this study is small.

The different mechanisms that may account for drug resistance have also been discussed. General endocrine resistance emerges when the tumour is originally hormone independent or becomes so especially with oestrogen receptor (ER) negative tumours and such cancers are unlikely to respond to aromatase inhibitors. Cellular heterogeneity with regard to hormone receptors and sensitivity has been documented [Hamon JT] although clinical observations are not totally compatible with differential clonal destruction. Thus if successful treatment with aromatase inhibitors were associated with selective destruction of oestrogen receptor positive cells and relapse associated with emergence of negative clones, oestrogen receptor content should fall. On the contrary, sequential analysis of tumours from patients treated with aminoglutethimide have shown that in majority oestrogen receptor (ER) levels following response are similar to before treatment. However all samples used in this study were oestrogen rich tissues. Oestrogen receptor status following treatment was not measured as this was beyond the aim of this thesis.

Experience with chemotherapeutic drugs suggests either the presence of efflux pumps or metabolic activity which reduces the effective levels of drug within the target cell [Juranka PF]. Clear evidence for such allied phenomena in aromatase inhibitors resistance is lacking. It is possible that in individual patients, hormone escape could contribute to tumour regrowth. If this is true more effective measures to suppress oestrogen levels should be beneficial.

Exvivo studies show that aromatase in a subset of tumour appears resistant to type I inhibitor formestane [Reed MJ]. Interestingly resistance can also be demonstrated invitro. Tumour homogenates were incubated with increasing concentration of 4

hydroxy androstenedione and a type II inhibitor (aminoglutethimide). Certain tumours showed classic sensitivity to both type of inhibitors while others displayed resistance to 4-hydroxy androstenedione while remaining sensitive to type II inhibitor. Similar differential resistance/sensitivity has been shown in site mutagenesis studies of aromatase enzyme [Kadohama N] but the particular causative mutations have not been shown to be present in clinical specimens [Sourdaine MG].

Acquired resistance after successful treatment with hormonal therapy may also result from emergence of hormone resistant clones of tumour cells, which may have been present from the initiation of therapy. Genetic alterations may be responsible for failure to respond and molecular analysis of the gene in these tumours may provide supportive evidence. This was not carried out as it was beyond the scope of our study but would provide a valuable insight for investigation in future. Structure function studies have been performed in which site mutation, have been introduced into cDNA encoding for aromatase [Kadohama N]. One point mutation has shown to decrease sensitivity to 4 hydroxystenedione without changing itself. This phenotype has some relevance to breast cancer as a subset of tumours appear to have aromatase activity which is insensitive the inhibitory effects of 4 hydroxyandrostenedione while maintaining sensitivity to aminoglutethimide [Miller WR, 1997]. Sourdaine et al failed to provide such evidence and suggested that it may be due to other modifications.

Thus in this study aromatase activity during neoadjuvant therapy has been useful in predicting response to treatment in malignant tissues while variable effect has been observed in nonmalignant tissues.

## **7. Breast adipose tissue on treatment with letrozole – polymerase chain reaction [PCR]**

The object of the present study was to determine the distribution of various exon-specific transcripts in breast adipose tissues obtained before and following treatment with letrozole (2.5 mg) for 3 months, as this would enable to understand the nature of the factors regulating aromatase expression following treatment. To achieve this, we employed competitive RT-PCR, utilising an internal standard for each exon-specific transcript of the CYP19 gene, as well as for the coding region, to evaluate total CYP19 gene transcripts.

Aromatase gene expression was studied in 6 samples of breast adipose tissue obtained before and following treatment with letrozole (2.5 mg] for 3 months. Total RNA was isolated using the Guanidine-Thiocyanate method as described before and purity checked by spectrophotometry and electrophoresis. Reverse transcription (RT) was performed with oligo-dT primers using a Promega kit in accordance with manufacturer's recommendation. Efficiency of reverse transcription (RT) was verified by polymerase chain reaction (PCR) with primers to GADPH gene. In all instances, a nested design of PCR was used. Expression of coding part of aromatase mRNA was verified examining the regions across exons II and III. The four most common variants of the untranslated and alternatively spliced first exon were also studied - I.1, I.3, I.4 and PII variants. The results are shown in **table – 17**.

Using primers on the coding region of aromatase, no product was detected after the first round of PCR (40 cycles) but following a second round of PCR (with nested primers)



products were observed in all the 6 samples studied before and following treatment with letrozole. The major band was of expected size (393bp) (**fig 17**).

In terms of RT-PCR for transcripts of the non-coding exon I, one or more product was found in all the 6 samples studied before and after treatment. The coding part of aromatase gene was expressed in all the samples studied. P.II, 1.3 and 1.4 variants of the 1<sup>st</sup> exon were detected in most cases before and following treatment with letrozole.













I.3 and P.II variants were detected in all the pre and post treated samples studied. The I.1 variant was seen only in post treated sample 2. The I.4 variant was observed in all the samples except in post treated sample 3. This meant that there could be possible induction of exon I variant I.1 and I.4 following treatment with letrozole in samples 2 and 3. To confirm this, the nested PCR was repeated using samples 2 and 3 and the results are shown in **table – 18**.

The induction of exon I variant I.1 and I.4 could not be reproduced using samples 2 and 3. Thus in patients 2 and 3, treatment with letrozole apparently induced expression of a previously unused variant of the 1<sup>st</sup> exon although this result was not reproducible. This discrepancy could be caused by the low levels of aromatase in adipose tissue and this challenges the current methodology of using nested PCR for detection of mRNA. Data on expression of the coding part of aromatase gene (exons II and III) also confirms the presence of aromatase mRNA in human adipose tissue. The minor non-specific products and the lack of consistency of expression in some samples probably reflect the difficulty in selective amplification of extremely low abundant template from total RNA samples from the tissue. Low abundance of the specific products and appearance of non-specific minor PCR







products have also been reported for extragonadal aromatase expression in other peripheral tissues [Harada N 1990, Koos RD 1993].

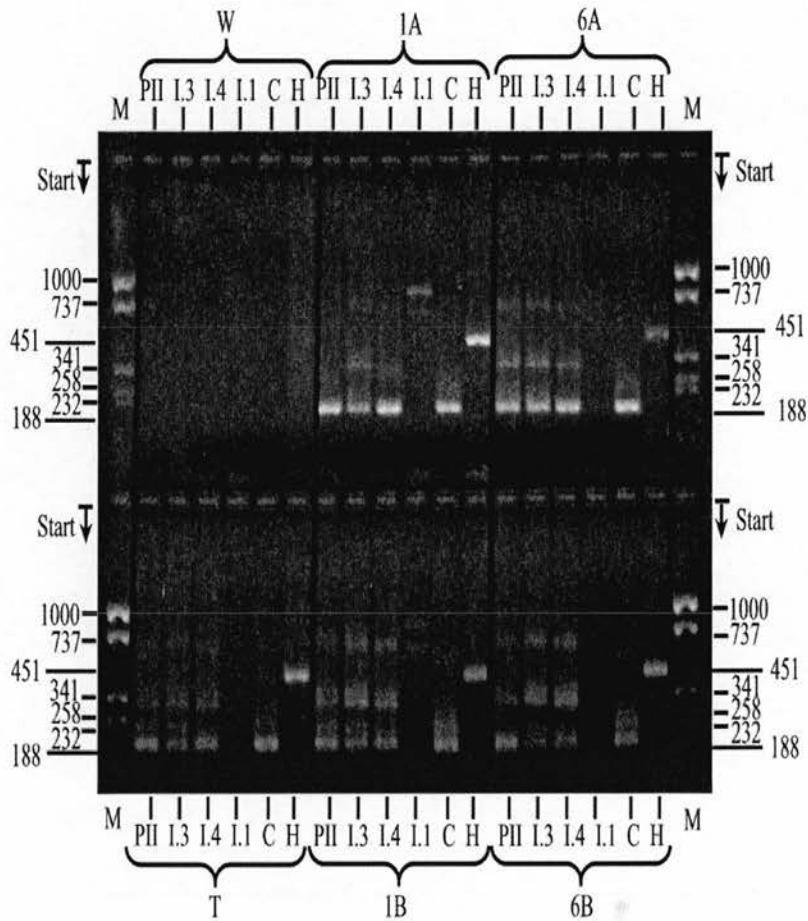
**Table-17: mRNA Expression [RT- nested PCR] of aromatase in adipose tissue before and following treatment with letrozole**

Patients' code	Sample	PII	1.3	1.4	1.1	Coding part (II-III exons)	Fragment of the gel photo
1	Pre	+	+	+	-	+	
	Post	+	+	+	-	+	
2	Pre	+	+	+	-	+	
	Post	+	+	+	+	+	
3	Pre	-	+	+	-	+	
	Post	+	+	+	-	+	
4	Pre	+	+	+	-	+	
	Post	+	+	+	-	+	
5	Pre	+	+	+	-	+	
	Post	+	+	+	-	+	
6	Pre	+	+	+	-	+	
	Post	+	+	+	-	+	

**Table -18: mRNA Expression [RT- nested PCR] of aromatase in adipose tissue before and following treatment with letrozole [experiment repeated to assess reproducibility]**

Patients' code	Sample	PII	1.3	1.4	1.1	Coding part (II-III exons)	Fragment of the gel photo
2	Pre	+	+	+	-	+	
	Post	+	+	+	-	+	
3	Pre	+	+	+	-	+	
	Post	+	+	+	-	+	

**Fig 17: Nested PCR - demonstrating samples 1 and 6 before and following treatment with letrozole**



- 1 A and 6A- breast adipose tissues before treatment
- 1 B and 6B- breast adipose tissues after treatment.
- Specific products are visible - the major bands for exons PII, 1.3, 1.4 and C in the samples shown. No product - seen in the negative control (W sample).
- In all other samples signals seen - positive controls (H lines).  
GAPDH- gene -positive control: H - product for GAPDH gene (451bp, positive control): T - aromatase- positive tumour (another positive control).
- Expected PCR products size: PII – 393 bp, 1.3 - 390 bp, 1.4 - 383 bp, I.I - 425 bp, C - 305 bp and H - 451 bp.

## **Treatment of breast adipose tissue with letrozole – using PCR**

The aromatase gene consists of 10 exons. Several alternative regions located in the 5' area of the gene are used as the exon I in different tissues. This provides mechanism for tissue-specific regulation of aromatase transcription because different variants of the 1<sup>st</sup> exon are controlled by different promoters. Exon 1.4 has promoter containing a glucocorticoid-responsive element but 1.3 and P.II variants are controlled by cAMP dependent mechanisms. At the same time, the start of translation is located in the second exon [Harada N, 1990b]. This means that the alternative splicing of the exon I affects the gene regulation only; whereas the coding part of the mRNA and protein structure does not depend on the exon I selection. Thus whilst there is a single protein for aromatase, multiple variants of RNA transcripts exist, differing according to the nature of untranslated exon I .

Use of Polymerase Chain Reaction (PCR) technology has allowed for determination of expression of P-450AROM in samples of breast adipose tissue before and following treatment with letrozole. In terms of the exon I usage in adipose tissue, this study suggests that the aromatase mRNA may be derived from different promoters. Such features are similar to those reported for aromatase gene activity in other peripheral tissues [Harada N, 1993a]. In the present work the activity of I.I ("placental" or Ia) variant, 1.4 ("adipose" or Ib), 1.3 and P.II ("ovarian" or Ic and Id) variants of the exon I were studied. The pattern of the exon I usage, found in the present study for adipose tissue, is similar to the pattern reported for adipose tissue by others: 1.4, 1.3 and P.II with prevalence of 1.4 exon [A garwal V, 1997]. In some cases (patients 2 and 3)

treatment with letrozole apparently induced activity of previously un-used variants of the 1<sup>st</sup> exon, although this result was not reproducible.

## DISCUSSION

CYP 19 gene and aromatase activity is subject to complex hormonal regulation, which is mediated in part by alternative use of tissue specific promoters of the CYP19 gene [Mahendroo M]. The human gene is revealed to contain multiple variants of exon 1, which are tissue specifically, selected [Means GD, 1989]. Each exon 1 is flanked with a unique promoter region, which may explain tissue-specific transcription. P450 is stimulated upstream of untranslated exons 1.4 and are regulated in a positive fashion by glucorticoids and growth factors present in serum [Zhao Y, 1996]. In contrast, transcripts containing exon 1.4 are absent when cells are maintained in the presence of cyclic AMP derivatives and when fibroblasts are placed in culture, the proportions of these exon specific transcripts depend on the stimulatory factors present in the culture medium [Bulun SE, 1994a]. Thus when aromatase activity is stimulated by members of class I cytokines such as interleukin 6 in the presence of glucorticoids, the transcripts present are derived from promoter I.4 and when activity is stimulated by dibutyryl cAMP the transcripts present are those specific for PII and I.3 [Agarwal VR, 1996]. Activity of aromatase P450 arom is aberrantly increased in adipose fibroblasts surrounding breast carcinomas, giving rise to proliferation of malignant cells [Zhou J, 2001]. Attempts were made in this project to study the exon specific transcripts and the changes in fibroblast cell lines following treatment with aromatase inhibitors in the presence and absence of glucorticoids and dibutyryl cAMP. However, the experiments failed to detect any changes due to very low level of mRNA in the fibroblast cells. Hence, development of new techniques in the future to isolate low levels of aromatase

activity in mammary fibroblasts cell cultures would be useful. This would lead to better understanding of molecular changes underlying the changes in aromatase activity in cell culture models, which could be translated into clinical practice.

Aromatase mRNA in normal breast tissues is mainly transcribed from exon 1b (I.4) of the gene. However, in breast cancer tissues, it is often transcribed from exon 1c/1d (I.3/PII). Such a switching from exon 1b to exon 1c/1d has often been found concomitantly with elevated levels of aromatase mRNA [Harada N, 1999]. Regulation of aromatase in breast adipose tissue becomes abnormal during carcinogenesis due to switching of exons I and promoters, resulting in overproduction of oestrogens under control of a new promoter [Harada N, 1995].

To explore this effect aromatase messenger ribonucleic acid (mRNA) and the type of multiple exons 1 of the human aromatase gene transcribed in breast adipose tissues before and after treatment with letrozole (2.5mg) were studied. The four most common variants of the untranslated (I.1/a placental, I.4/b adipose and PII/ 1c/d ovarian variants) and alternatively spliced first exons were analysed. No product was detected after the first round of PCR but following a second round of PCR (with nested primers), products were observed. Analysis of P450 arom transcripts in samples of breast adipose tissue has previously revealed that in addition to exon I.4 specific transcripts, promoter II specific as well as exon I.3 specific transcripts are present, albeit in low copy number [Mahendroo M]. Thus, no products were detected after the first round of PCR and this may due to the low quantities of mRNA transcripts and aromatase activity. However, despite the low level of P450 arom mRNA and aromatase activity, it is possible, that local biosynthesis in breast cancers may be of physiological and clinical significance.



Therefore, development of new techniques for the quantification of such low aromatase activity would be highly desirable in future.

Following nested PCR for transcripts of the non-coding exon I, one or more product was found in all the samples studied before and after treatment. The coding part of aromatase gene was expressed in all the samples studied. Therefore, this study demonstrates the presence of mRNA transcripts for aromatase in human adipose tissue.

It is known that I.4 promoter contains a glucocorticoid-responsive element but I.3 and P II variants are controlled by cAMP-dependent mechanism. Exons I.3 and PII are the two major exons present in aromatase mRNA isolated from breast tumours as detected by performing RT-PCR analyses [Zhou C, 1995; Bulun SE, 2003]. Results, suggests that promoters I.3 and II are the major promoters directing aromatase activity in breast cancer and surrounding stromal cells and fibroblasts [Chen S, 1999]. PII, I.3 and I.4 variants of the 1<sup>st</sup> exon were detected in most cases before and following treatment with letrozole in this study. Two patients (2 and 3) demonstrated possible induction of the new exons after treatment with letrozole although it was not reproducible. These findings, suggests that there is a may be a switch in the regulatory mechanism of aromatase activity from normal breast tissue to cancerous tissue.

Therefore, this study demonstrates the presence of mRNA transcripts for aromatase in human adipose tissue. Level of aromatase mRNA in adipose tissue is low. Neither conventional (“end-point”) nor nested PCR can be used for quantitative measurement of aromatase transcripts in this tissue.

Although aromatase activity and level of mRNA activity are low, such small activity can be meaningful, because low activity may be compensated by bulk of the tissue in the

body. Switching of tissue-specific exons 1 though not totally reproducible in this study, may affect strict regulation, leading to abnormal activity of the aromatase, which could account for paradoxical effects/resistance in long-term use of aromatase inhibitors

## CONCLUSION

This thesis has successfully examined the effects of different aromatase inhibitors on aromatase activity and assessed the activity of aromatase in response to endocrine therapy. It has confirmed that the aromatase inhibitors are potent and effective in inhibiting aromatase activity. Indeed, it is one of the fewer studies to report the effects of different aromatase inhibitors using the cell culture model invitro.

The effects of antiaromatase drugs on aromatase in fibroblast cell culture model would be useful in defining the role of aromatase inhibitors when translated into clinical practice. The steroidal aromatase inhibitors have produced persistent inhibitory effect in all circumstances (both on preincubation and incubation) while paradoxical stimulatory effects have been observed with type II inhibitors in certain circumstances (on preincubation). The relevance of this type of response would be useful in determining the sequence of use of aromatase inhibitors in the clinical setting. Interestingly at least at one concentration, all the type II inhibitors produced a paradoxical increase in aromatase activity. This induction of aromatase activity can result from enhanced transcription of the aromatase gene and or stabilisation of the aromatase protein. It may be postulated that increased levels of aromatase protein following chronic treatment with type II inhibitors could lead to break-through of oestrogen synthesis and renewed tumour growth. It also indicates the possibility that long-term use of type II inhibitors can result in hormonal escape and drug resistance. Hence, it would be useful to use type II inhibitors such as anastrozole or letrozole followed by type I inhibitors in sequence. However, this effect when translated into clinical practice can only be determined after significant clinical

follow up of patients treated on long-term aromatase inhibitors. The results also suggest that the type II agents - letrozole, anastrozole, fadrazole and aminoglutethimide are reversible, where as type I agents - exemestane and formestane are irreversible.

An accurate measurement of aromatase activity allows better understanding of the response to endocrine therapy at a cellular level. The changes in aromatase activity revealed interesting trends with almost total suppression of aromatase activity following treatment in tumour tissues and a variable effect in nonmalignant tissues. Surprisingly so far, no positive correlation has been linked to recurrence or survival. Nevertheless, the significant positive correlations between the degree of response and treatment activity are potentially important in understanding the mode of action of aromatase inhibitors and should be examined further.

The examination of changes in aromatase activity before and after treatment in nonmalignant tissues has raised the possibility of alteration of activity following treatment. However, the relatively few patients receiving primary endocrine therapy has made it difficult to draw any useful conclusion from this section of work. Because of the potential implication of this finding, further work with the aromatase activity needs to be undertaken. Selection for endocrine therapy requires the identification of markers that accurately predict response/resistance. Molecular profiling may offer the opportunity to distinguish between clinically responsive and non-responsive tumors and may provide important information about the heterogeneity of endocrine resistance in the future.

Intratumoral aromatase is an important therapeutic target for the treatment of post-menopausal oestrogen-dependent breast cancers. Therefore, reliable methods should be developed for routine application for the detection of intratumoral aromatase.

Biochemical assays used in determining aromatase inhibitors are cumbersome and need large amount of tissue. Development of appropriate antibodies to detect and assess aromatase would avoid this problem in future. Hence, further work would be required of estimating aromatase activity through other methods. The potential promise of developing new techniques to assess aromatase at the molecular level would be rewarding. The limiting factors include quality, quantity and the low RNA activity in these tissues.

The new generation of aromatase inhibitors, are potent and specific inhibitors of aromatase peripherally and within the breast. The inhibitors differ with regard to structure and mode of interaction with the aromatase enzyme and this may account for their effects. The work done in this thesis has demonstrated unusual effects of aromatase inhibitors, which has to be confirmed from the long-term use of aromatase inhibitors and from clinical trials.

The aromatase inhibitors have emerged as an effective and safe alternative to tamoxifen in the adjuvant setting. Given the proven efficacy of AIs in recent clinical trials, the remaining questions include definitive sequencing strategy, timing, and duration of use. Ongoing trials include head-to-head comparisons between the AIs in early-stage breast cancer; the results of these trials should further optimise the use of AIs. New studies in both the preclinical and clinical settings will explore the role of the steroidal aromatase inhibitors such as exemestane in comparison to the nonsteroidal aromatase inhibitors such as letrozole and anastrozole and may prove of considerable interest. Results from trials comparing steroidal and nonsteroidal AIs will determine whether meaningful clinical differences in efficacy or adverse events exist between the classes of AI. Studies

concerning lack of cross-resistance and optimum sequencing will also provide useful data over the next few years.

Thus the new generation of aromatase inhibitors, provide not only new approaches to treatment but also help in instigating the contribution of aromatase to tumour induction and proliferation. This study has demonstrated the effects of aromatase inhibitors on aromatase in response and resistance to therapy. It forms the basis for more detailed work in future to optimise the use of currently available aromatase inhibitors in the hormonal treatment of breast cancer.

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## ABBREVIATIONS USED IN TEXT

Cyclic AMP	cyclic adenosine monophosphate
UK	United Kingdom
mRNA	messenger ribonucleic acid
DNA	deoxyribonucleic acid
ER	oestrogen receptor
PR	progesterone receptor
EGF	epidermal growth factor
IGF	insulin-like growth factor
FGF	fibroblast growth factor
TGF	transforming growth factor
LHRH	luteinizing hormone-releasing hormone
FSH	follicle stimulating hormone
LH	luteinizing hormone
NATO	Nolvadex Adjuvant Trial Organisation
EBCTCG	Early Breast Cancer Trialist's Collaborative Group
NSABP	National Surgical Adjuvant Breast and Bowel Project
SERMS	selective oestrogen receptor modulators
PGE	prostaglandin
RACE	rapid amplification of cDNA ends
GAS	gamma activating sequence
STAT	signal transducer and activator of the transcription

MA	megestrol acetate
PBS	phosphate buffered saline
MEM	minimal essential medium
FCS	foetal calf serum
DMSO	dimethyl sulphoxide
DEPC	diethyl pyrocarbonate
RT	reverse transcription
PCR	polymerase chain reaction
Dpm	disintegrations per minute
$^3\text{H}$	Tritium
HR	Hazard ratio

## APPENDICES

### Appendix to Materials and Methods

## MATERIALS

### Cell experiments

Minimal Essential Medium (alpha MEM)	#22571-020	LIFE TECHNOLOGIES [UK]
Foetal Bovine Serum (heat inactivated)	#F9665	SIGMA ALDRICH [UK]
Dulbecco's Phosphate Buffered Saline	#D5652	SIGMA ALDRICH [UK]
Penicillin & Streptomycin (10,000 units/10mg)	#PO781	SIGMA ALDRICH [UK]
Culture plates - 6 well plates (polystyrene)	#C3516	MACKAY AND LYNN [UK]
Collagenase type VIII	#C2139	SIGMA ALDRICH [UK]
Syringe Filter 0.2µm	#S2052-025	MACKAY AND LYNN [UK]
Pasette 1ml (plastic)	#Z350591	SIGMA ALDRICH [UK]
Glass pipettes -10ml	#PP280-53	STERILIN [UK]
Glass test tubes (ground socket)	# MF24/1	MACKAY & LYNN [UK]
Petri dishes	# P3924	MACKAY AND LYNN [UK]
Trypsin EDTA solution	#T4299	SIGMA ALDRICH [UK]
Bio-freeze vials	#C4166	COSTAR [UK]
Chloroform CDG	# RH2009	RATHBURN CHEMICALS [UK]

1 $\beta$ - <sup>3</sup> H Androst-4ene-3, 17-dione	NET-926	DUPONT [UK]
4-Androst-4ene-3, 17-dione	#A9630	SIGMA [UK]
Charcoal (activated USP)	#C7606	SIGMA [UK]
New-born calf serum	# 06000010M	GIBCO [UK]
Liquid scintillant	# NE260	NUCLEAR ENTERPRISES UK]
Tri-carb 1900 CA liquid	# 1900CA	PACKARD INSTRUMENTS
Paper boats		
30mls	# BD 753-62	MACKAY & LYNN [UK]
100mls	# BD 753-64	MACKAY & LYNN [UK]
Melamine tray	# 686-006	JENCONS-PLS
Centrifuge tube (50mls)	# 430829	CORNING [USA]
Centrifuge tube (15mls)	# 430791	CORNING [USA]

## Tissue experiments

Adenosine 5' Triphosphate [ATP]	#A7699	SIGMA [UK]
$\beta$ Nicotinamide adenine dinucleotide [NAD]	#N0505	SIGMA [UK]
Nicotinamide adenine dinucleotide phosphate [NADP]	# N1636	SIGMA [UK]
Glucose 6 phosphate	#G8878	SIGMA [UK]
Glucose 6 phosphate dehydrogenase	#N346774-5000	SIGMA [UK]
Androst-4-ene-3, 17-dione		SIGMA [UK]
1 $\beta$ - <sup>3</sup> H- Androst-4-ene-3, 17-dione	#NET 926	DUPONT [UK]
Magnesium chloride	# 3221	SIGMA [UK]
Charcoal	# C5260	SIGMA [UK]
Disodium hypophosphate dihydrate	#7792-85-6	SIGMA [UK]
Monosodium hypophosphate dihydrate	#7558-80-7	SIGMA [UK]
Chloroform GDG	#32001-31-0	RATHBURN CHEMICAL [UK]
1 $\beta$ - <sup>3</sup> H Androst-4ene-3,17-dione	#NET-926	DUPONT [UK]
4-Androst-4ene-3, 17-dione	#AA9630	SIGMA [UK]
Charcoal	#C5260	SIGMA [UK]
Liquid scintillant	#NE260	NUCLEAR ENTERPRISES [UK]
Tri-carb liquid scintillation analyser	# 1900 CA	PACKARD INSTRUMENTS [US]

### Collagenase

Collagenase dissolved at 1mg/ml of alpha MEM.

Filtered through a millex-GS sterile 0.22  $\mu\text{m}$  filter and stored in 5ml aliquots at  $-20^{\circ}\text{C}$ .

### Freezing mixture

90% New-born calf serum [heat inactivated]

10% Dimethyl sulphoxide

### Phosphate buffer

Phosphate buffer was prepared as follows:

Phosphate buffer [0.1 M, pH 7.4 containing 5mM  $\text{MgCl}_2$ ]

A. 0.2M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

8.90g/250ml

B. 0.2M  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

3.12g/100ml

202.5ml of solution A }

47.5 ml of solution B } to 500 ml

509.5 mg of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  }

### Precursor mixture

10  $\mu\text{l}$  of 6Glucose phosphate dehydrogenase

10  $\mu\text{l}$  of 1  $\beta$  3H androst-4-ene-3, 17- dione

100  $\mu\text{l}$  of inhibitor [letrozole]

0.5 ml of cofactor mixture

0.5ml of particulate fraction

0.5ml of buffer to the blanks for the particulate fraction

80  $\mu\text{l}$  of cold 1  $\beta$  3H androst-4-ene-3, 17- dione

The cofactors were prepared as detailed below:

Cofactors	mg/ml
• Adenosine 5'Triphosphate [ATP], 22 mM	13.31
• $\beta$ Nicotinamide adenine dinucleotide [NAD], 22 mM	14.59
• $\beta$ Nicotinamide adenine dinucleotide phosphate [NADP], 22 mM	18.66
• Nicotinamide, 100mM	13.43
• Glucose 6 phosphate, 110 mM	39.39
• Glucose 6 phosphate dehydrogenase	250 units/ml of buffer

SOLUTION	VOLUME / INCUBATION	FINAL
ATP [22 mM]	100 $\mu$ l	2 mM
NAD 22mM]	100 $\mu$ l	2 mM
NADP [22mM]	100 $\mu$ l	2 mM
NICOTINAMIDE [110mM]	100 $\mu$ l	10 mM
GLUCOSE 6 PHOPHATE [110mM]	100 $\mu$ l	10 mM
G6PDH [250 units/ml]	10 $\mu$ l	2.3
1 $\beta$ -3H- ANDROST-4-ENE-3,17-DIONE [100uCi/ml, 3.65nmol/ml]	10 $\mu$ l	1 $\mu$ Ci
ANDROST-4-ENE-3,17-DIONE [0.794 nmol/ml]	<u>80 <math>\mu</math>l</u>	0.1nmol
	<u>600 <math>\mu</math>l</u>	



### **Charcoal suspension**

Charcoal 5g in 100ml buffer containing 0.05% Tween 80 [v/v].

Charcoal pellets were prepared by centrifuging 1ml [2000g for 15 min] in 12mmX 75 mm glass tubes and aspirating the supernatant.

### **Androst-4-ene-3, 17- dione**

1  $\beta$  3H androst-4-ene-3, 17-dione, 100uci/ml 3.61nmol/ml.

Purified by thin layer chromatography on silica gel using chloroform: acetone [185:15].

Dissolved in ethanol to a concentration of 100uCi/ml. Duplicate aliquots [10 $\mu$ l] were transferred at the time of addition of the titrated to the incubation mixture, into 60x27mm glass counting vials containing 10ml NE260 liquid scintillator for measurement of the specific activity of the precursor.

### **Androst-4-ene-3, 17- dione**

Androst-4-ene-3, 17-dione, 22ng/ml in buffer.

Prepared from a 1mg/ml ethanolic solution as follows:

1. Diluted 0.1 ml to 10 ml with buffer to give a 10  $\mu$ g/ml solution
2. Diluted 0.1 ml of solution [1] to 4.4 ml with buffer to give a 227ng/ml solution.

### **Reaction Mixture**

Reaction Mixture was prepared as detailed below.

10  $\mu$ l of 1 $\beta$ -3H androst -4ene-3, 1-17 dione [2 $\mu$  Ci]

90  $\mu$ l of cold anrost-4ene-3, 7 dione

3.9 ml of alpha MEM containing no FCS

### **Charcoal suspension preparation**

Charcoal [sigma C5260]: 5g in 100ml buffer containing 0.05% Tween 80 [v/v].

Charcoal pellets were prepared by centrifuging 1ml [2000g for 15 min] in 12mmX 75mm glass tubes and aspirating the supernatant.

### **RNA mix**

0.5 µl of rRNasin ribonuclease inhibitor [40units/µl]

4 µl of 25 mM MgCL<sub>2</sub>

2 µl of 10 X reverse transcription buffer

2 µl of 10mM dNTP mixture

1 µl of oligo [dT] [0.5 µl]

0.6 µl of AMV reverse transcriptase [HC: 25 units / µl]

Made up to a final volume of 20 µl using ultrapure water.

## **APPENDIX – 2**

### **PUBLICATIONS**

#### **Book – Chapter**

Miller WR, Vidya R, Mullen P, Dixon JM. Induction and suppression of aromatase by inhibitors. Aromatase and Breast Cancer. Ed WR Miller, RJ Santen, New York, Marcel Dekker 2000: 213-25

#### **Abstracts**

1. Vidya R, Dixon JM, Dean I, Miller WR. Paradoxical effects of Aromatase inhibitors: Increased activity of target enzyme following prior exposure to some but not all aromatase inhibitors. Breast Cancer Res Treat 2000; 58 (Suppl 1):74
2. Howie AF, Vidya R, Mason JI and Miller WR. Nature of aromatase transcripts in breast cancer tissues. Brit J of Cancer 1999; 80 (Suppl 2):33

#### **Presentation - Posters**

1. Vidya R, Dixon JM, Evans DB, Miller WR. Paradoxical effects of Aromatase inhibitors: Increased activity of target enzyme following prior exposure to some but not all aromatase inhibitors. 23<sup>rd</sup> Annual San Antonio Breast Cancer Symposium, December 2000.
2. Vidya R, Dixon JM, Evans DB, Miller WR. Effects of aromatase inhibitors on aromatase activity in mammary adipose tissue. International Aromatase Conference, Cairns, Australia, November 2000.
3. Howie AF, Vidya R, Mason JI, Miller WR. Nature of aromatase transcripts in breast cancer tissues. British Cancer Research Meeting, July 1999.